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CLINICAL LABORATORY DIAGNOSIS

"Sans doute, le laboratoire est loin de donner des diagnostics tout faits, et ce serait une grave erreur que de les lui demander; il ne supprime pas l'étude clinique directe du malade, mais il la complète, l'éclaire toujours, la rectifie souvent."

A. CHAUFFARD.

The laboratory is, of course, far from being able to give complete diagnoses, and it would be a great mistake to expect it to do so; it does not take the place of the direct clinical study of the patient, but completes the latter, clarifies it always, clinches the diagnosis frequently.

CLINICAL LABORATORY DIAGNOSIS

DESIGNED FOR THE USE OF STUDENTS AND PRACTITIONERS OF MEDICINE

BY

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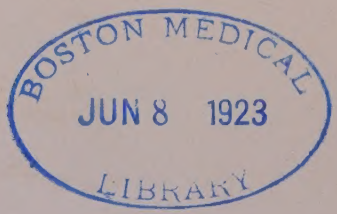

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TO THE MEMORY OF

CHRISTIAN R. HOLMES

PREFACE

Clinical pathology has undergone a rapid development in the last twenty years. Formerly, it was often necessary to urge the importance of laboratory procedures in the routine examination of patients, but now, fortunately, the need of such aid in the study of disease is recognized by practitioners; and with the more general use of these tests, their true value has become widely appreciated.

No single method of examining a patient can lead to a diagnosis. History, physical findings and laboratory data constitute a triad which must be worked out carefully and completely before one can feel that one's duty to the patient has been fully discharged. The beginner is apt to expect too much of the laboratory examinations; clinical data are always equally essential in arriving at a diagnosis. It is not enough, for example, to know that a patient has malarial parasites in his blood; it is of equal or even greater, importance to know how he is reacting to the plasmodia. So, too, a positive Wassermann reaction is meaningless, unless the history and physical findings are available—and even then, the positive complement fixation may be difficult to interpret. The successful practitioner is the one who does his own laboratory work or who actively supervises it and himself interprets it in the light of the clinical data.

The author has attempted, in the present volume, to give the various laboratory procedures employed in the study of patients, together with data to assist in their interpretation. Lists of the diseases in which an abnormal finding may occur have been given. These tabulations were chosen, after much consideration and discussion with colleagues and other friends, in preference to more extended presentations, for several reasons, among which may be mentioned (1) conservation of the time of the worker, who, in a moment, may run through a list of diseases instead of reading several pages to gain much the same information; (2) a desire to have the volume so small that students may use it conveniently in wards and laboratories; and (3) the hope that the tabulations may prove useful to instructors as outlines for class lectures.

The following works have been consulted freely: Castellani and Chalmers' "Manual of Tropical Medicine," Laroche's "Examens de Laboratoire du Médecin Practicien," Hoppe-Seyler's "Handbuch der chemischen Analyse," Mathews' "Physiological Chemistry," Zinsser's "Textbook of Bacteriology," Neveu-Lemaire's "Précis de Parasitologie Humaine," Hammarsten's "Lehrbuch der physiologischen Chemie," Blanchard's "Traité de Zoologie Médicale," Rosenau's "Preventive Medicine and Hygiene," and Naegeli's "Blutkrankheiten und Blutdiagnostik." To the more recent literature, direct reference has been made in the form of footnotes. In all instances, the writer has endeavored to give proper credit to authors.

To his colleagues in the College of Medicine of the University of Cincinnati, and, more particularly, to his associates of the Medical Clinic of the Cincinnati General Hospital, the writer owes a debt of gratitude which he can never hope to repay. He is under particular obligations to Dr. Mark A. Brown for many helpful criticisms and suggestions; to Dr. J. C. Oliver, recently dean of the College, for encouragement in many directions; to Dr. Wm. B. Wherry for critical aid in the matter dealing with bacteriology and immune reactions; to Dr. Hiram B. Weiss for indicating the need of some of the matter; to Drs. Raphael Isaacs and D. S. Hachen of the Resident Medical Staff of the Hospital, especially for the preparation of the material dealing with chemical analysis of the blood, and to Dr. Isaacs for his generosity in permitting the use of some (as yet) unpublished observations; to Mr. Robert C. Walker for helpful criticism of parts of the manuscript; and to Miss Bertha DeMar for invaluable assistance in the preparation of the manuscript and in other ways. And to a colleague who has gone to his Maker, Christian R. Holmes, the modern Father of Medicine in Cincinnati, dean, builder of the new Cincinnati General Hospital and of the new College of Medicine of the University of Cincinnati, peerless leader, wise counselor, devoted friend, it is the rare privilege of the author to pay honor on the dedicatory page.

R. S. Morris

Cincinnati

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CHART FOR LABORATORY DATA

Name.....

(Cincinnati General Hospital)

BLOOD EXAMINATION

Date and Time	Examined By	White Cells	Red Cells	Hemoglobin (% Method)	Color Index	Number of Cells Counted in Differential	% Ly.	% L. M. & Tr.	% Pm. N.	% Pm. E.	% Pm. B.	Pathological Leukocytes and Red Cells	Other Data (Stain Used)

URINE EXAMINATION

Date and Time	Examined By	Type of Spec. (Fresh; 24 hours)	Color and Appearance	Reaction	Sp. Gr.	Quantity	Alb.	Sugar	Microscopic	Other Data

GASTRIC CONTENTS

Date and Time	Examined By	Meal	Time in Stomach	Quantity Aspirated	Free HCl	Total Acidity	Lactic Acid	Microscopic	Other Data

STOOL EXAMINATION

Date and Time	Examined By	Color	Form	Mucus	Blood	Parasites and Ova	Microscopic	Other Data

SPUTUM

Date and Time	Examined By	Character	Quantity	Odor	Elastic Tissue	Bacteria	Type of Leukocytes	Other Data

CEREBRO SPINAL FLUID

Date and Time	Examined By	Appearance	Quantity	Color	Globulin	Wassermann	Cells (No. Type)	Bacteria	Other Data

PUNCTURE FLUIDS (Pleural, Pericardial, Peritoneal)

Date and Time	Examined By	Source and Appearance	Quantity	Color	Sp. Gr.	Protein per Liter	Cells	Bacteria	Other Data

BLOOD CULTURES, WIDALS, WASSERMANN TESTS

Date and Time	Examined By	

BLOOD CHEMISTRY

Date and Time	Examined By	Glucose 77–119 mg.	Non-Prot. N. 26–43 mg.	Urea Nitrogen 10–22 mg.	Uric Acid 1–4.5 mg.	Creatinin 1.2–2.5 mg.	Alkali Reserve 53–77 c. c.	Chlorides 450–500 mg.	Fats 440 mg.	Cholesterol 140–170 mg.	Other Data

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CLINICAL LABORATORY DIAGNOSIS

CHAPTER I

THE URINE

Collection of the Urine.—For chemical examination, as a general rule, the total amount of urine for the twenty-four hours should be preserved.[1] The reason for saving *all* the urine is that different voidings may vary greatly in their chemical composition. In the morning, for example, an albuminuric may excrete urine which is normal chemically, whereas specimens obtained after the patient has had more or less exercise may contain albumin. Thus, it becomes necessary that a mixture of all the urine passed during the twenty-four hours be obtained in order to avoid the possibility of error, for the examination of the early morning specimen alone, in the case just cited, would be entirely misleading. Special circumstances arise at times, nevertheless, which make it desirable to break the rule and to secure one or more voidings at special hours of the day. For the purpose of quantitative chemical analysis, it is, of course, absolutely essential to have the total amount of urine for twenty-four hours collected.

For *microscopic examination* a perfectly fresh specimen should always be insisted upon. The organized elements of a urinary sediment rapidly deteriorate, especially with high temperatures, so that within a few hours after the urine has been passed they may be unrecognizable, or completely disintegrated. When it is not possible to make an immediate examination, a preservative (thymol, toluol) should be added to the specimen, which is kept in an ice-chest as a further safeguard.

---

[1] In private practice, with ambulatory patients, this is often impossible. In such cases, samples of as many voidings as possible in the twenty-four hours should be saved and mixed.

Preservation of the Urine.—To all twelve-hour or twenty-four-hour specimens of urine, a preservative should be added to prevent decomposition through bacterial growth. The urine should also be kept on ice when possible. The receptacle for the urine must be perfectly cleaned and tightly stoppered.

1. *Toluol* (toluene) is, on the whole, one of the most satisfactory preservatives. It apparently interferes with none of the urinary tests. Bacterial growth is successfully inhibited by means of it. The one disadvantage in its use results from the fact that toluol floats on the urine; it is necessary to pipette or siphon the urine to obtain it without admixture of the preservative. The objection is a minor one. Diacetic acid may be preserved for weeks, whereas it disappears in a short time when other preservatives are used. In acidosis, therefore, toluol should be used as the preservative. Organized sediments are often beautifully preserved, but it must be remembered that casts or cells, becoming attached to droplets of toluol, rise to the surface and may be missed, when only a few are present; spontaneous sedimentation cannot be relied on if toluol is employed. The pipette used for withdrawing the sediment must be wiped to remove the toluol before placing the drop on a slide for examination.

2. *Chloroform* is the most generally used preservative for chemical work. It is a fairly strong reducing agent, and urine preserved with it must be boiled to drive it off before any of the reduction tests for sugar are performed. If the sediment is to be examined, care should be exercised to avoid drawing up chloroform with it.

3. *Thymol* is very satisfactory, and with it the formed elements of the urine are often very well preserved. As Weinberger [2] has shown, many urines to which thymol has been added give a positive Heller's test, though albumin be absent, a source of error which must be kept in mind. A positive test for bile may also be obtained after thymol preservation (Emerson).

Gum camphor and *formaldehyd* are used occasionally as preservatives. Formaldehyd, like chloroform, is a reducing agent. When available, toluol, chloroform, or thymol is to be preferred.

Macroscopic Examination of the Urine.—As a general rule, *normal, freshly voided urine* is perfectly *clear;* the same is true of the majority

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[2] Weinberger, W. "Thymol as a source of error in Heller's test for urinary protein." *Jour. A. M. A.*, 1909, LII, 1310.

of pathological urines. Occasionally, if the reaction of the urine be alkaline when voided, a turbidity may result from the precipitation of the phosphates and carbonates in the bladder, in the absence of a cystitis. Ordinarily, however, fresh urine, when cloudy or turbid, contains pathological ingredients, such as blood, pus, bacteria in large number, phosphates, bile salts, etc. Normal and pathological urines will become turbid and produce a macroscopic deposit, more or less abundant, if allowed to stand for some hours. Concentrated urine often furnishes an abundant precipitate of urates on cooling; the urates may be redissolved by warming the specimen. More frequently bacterial decomposition is the cause of the turbidity. Turbidity of the freshly voided specimen may be due to an excess of bile salts in the urine.[3]

The *nubecula* is a translucent cloud, composed chiefly of mucin (mucous threads) enmeshing epithelial or other cells, which forms in the urine a short time after it is passed.

Color.—The *color* of the urine is usually dependent on the quantity of water excreted in the twenty-four hours; the smaller the amount of urine the deeper the color, and *vice versa.* Normal urinary pigments in increased concentration or pathological pigments may lead to *abnormal coloration* of the urine (*see urobilin, bilirubin, hemoglobin, hematoporphyrin,* etc.). Following the administration of certain drugs, the color of the urine may be altered, the most striking change being the green color produced by methylene blue.

Quantity.—The *normal average amount* of urine for the twenty-four hours in this country is about 900 to 1,200 c.c. The limits of the normal are said to be 800 to 3,000 c.c. (Emerson). *In health* the quantity depends chiefly upon two factors, the amount of water consumed and the amount lost by perspiration. In disease the quantity of urine passed in twenty-four hours may be normal, increased, decreased, or nil.

Day and Night Urine.—In some instances, the urine is saved to advantage in twelve-hour periods, 8 A.M. to 8 P.M., and 8 P.M. to 8 A.M. *In health,* the ratio of the quantity of the day urine is to that of the night urine as $1:{}^1/_4$ or $1:{}^1/_3$, occasionally as $1:{}^1/_2$. With a total output of 500 c.c. or less, the volumes are nearly equal, and the ratios are of less significance. In normal adults who work at night and rest during the day, the ratios are reversed, the work period volume being three to four times that of the rest period volume.

---

[3] Oliver, S. F. Personal communication.

In *renal disease,* as shown by Edmunds,[4] Jones,[5] and others, the ratios may be reversed:

1. *In chronic interstitial nephritis,* the normal ratio is often reversed, and is uninfluenced by the ingestion of large amounts of fluid.
2. *In chronic parenchymatous nephritis,* if the output is small, the ratio determination has less value, though with total output of 800 c.c. or more, the disturbed ratio is found.
3. *In circulatory failure,* the day urine may be much less than the night urine; with improvement in the cardiac condition and the concomitant improvement in the circulation as a whole, and especially the circulation in the kidneys, there is a gradual return to the normal ratio.
4. In patients with chronic nephritis who *work at night,* the volume of the day or rest period is greater than, or equal to, that of the night or work period (Jones).
5. On a *protein-free diet* (with lessened quantity of nitrogenous waste-products to excrete), the normal ratio may be reëstablished (Jones).
6. The *alteration of the normal ratio* is one of the *earliest signs* of functional impairment of the kidney.

Polyuria (pathological increase in the quantity of urine in the twenty-four hours) is said to exist when the total daily excretion of urine exceeds 3,000 c.c. It is of significance only when more or less persistent, and is met with in:

1. *Diabetes mellitus,* from 3 to 5 liters in mild cases to 15 to 20 or even 25 liters in severe cases.
2. *Diabetes insipidus,* from 3 to 6 liters up to 43 liters.
3. During *convalescence* from fevers, not infrequently in convalescence from acute nephritis.
4. *Chronic interstitial nephritis.*

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[4] Edmunds, C. W. "Observations on the quantity of day and night urine." *N. Y. Med. Jour.,* 1904, LXXIX, 245.

[5] Jones, H. W. "The diagnostic value of volume ratio determinations of day to night urine." *Jour. A. M. A.,* 1922, LXXVIII, 477.

5. *Circulatory failure,* during stages of improvement, when transudates and edema fluid are being rapidly absorbed; also in *anemias* at times.
6. *Pleural, pericardial or peritoneal exudates* (*or transudates*), when rapidly absorbed.
7. *Transient polyurias* may be observed in connection with (*a*) *intermittent hydronephrosis* (Dietl's crises; kinked ureter); (*b*) *angina pectoris* following the attack; (*c*) *epilepsy* following the convulsion; (*d*) *hysteria;* (*e*) *psychical disturbances* (fright, etc.).

Anuria (failure to pass urine) may be renal or extra-renal in origin.

1. *Renal.* In *acute nephritis,* the amount is greatly diminished, and in extreme cases no urine (or less than 100 c.c.) may be secreted by the kidneys in twenty-four hours.
2. *Extra-renal.* These are classified as follows:[6]
   a. *Obstructive,* as in hypertrophy or tumor of the prostate, vesical calculus, urethral trauma.
   b. *Paralytic,* as in spinal cord lesions.
   c. *Reflex,* as in calculus in the pelvis or ureter of one kidney or after operation on one kidney, with reflex inhibition of secretion of the other kidney.
   d. *Prerenal,* from functional disturbances, as hysteria (succeeded by polyuria); from poisons, as phosphorus, turpentine, lead, ether, chloroform; in collapse; and often pre-agonal.

A *decreased quantity of urine* (*oliguria*) (that is, less than 800 c.c. daily) may be met with in:

1. Any of the *conditions causing anuria.*
2. *Chronic parenchymatous nephritis.*
3. *Circulatory failure.*
4. *Severe diarrheas.*
5. *Poisons,* such as bichlorid of mercury (at times, anuria).

---

[6] Emerson, C. P., *Clinical Diagnosis* (5th ed.), 1921, p. 92.

6. *Occupational,* as in persons working in very high temperatures, such as stokers. The excessive loss through the skin decreases the amount of fluid passed by the kidneys, unless compensated by increased intake.

REACTION OF URINE

The reaction of the urine is usually slightly *acid,* owing to the presence of an excess of dihydrogen (diacid) phosphates. An *amphoteric* reaction (red litmus turned blue and blue turned red) may be encountered, due to the fact that monosodium phosphate, an acid salt, may exist in the urine in conjunction with disodium phosphate, which is alkaline. An *alkaline* reaction is produced largely by an excess of alkaline phosphates and carbonates. That the salts are not the only factor in rendering a urine acid has been shown by Folin, who finds that at times nearly half of the acidity may be due to organic acids.

Litmus paper is used in testing the reaction of the urine. Unpreserved specimens, which have been allowed to stand for some time before testing, are often alkaline from ammoniacal fermentation produced by bacteria. The alkalinity in this case is differentiated from that due to fixed alkali by the odor, by the fact that on boiling the specimen the steam will turn blue a piece of moistened red litmus held in the neck of the test tube, or will cause a white frost of ammonium chlorid to appear on a glass rod, which has been dipped in hydrochloric acid. In disease the urine may be ammoniacal before it is voided.

The *total acidity* of the urine is increased:

1. *Protein diet.*
2. Severe *physical exertion.*
3. Many cases of *acidosis.*
4. *Poisoning* with *inorganic acids.*
5. Certain cases of *"neuralgia"* or neuralgic headaches. (In some cases correction of the hyperacidity is followed by relief of pain.)
6. At times in *young girls with symptoms of cystitis.*

Quantitative Determination of Urinary Acidity

For quantitative determination of the acidity the twenty-four-hour specimen is used. It is necessary to prevent decomposition by the addition of a preservative.

Folin's Method.[7]

Reagents:

$\frac{1}{10}$ sodium hydrate.[8]
0.5 per cent phenolphthalein in 50 per cent alcohol.
Potassium oxalate, neutral.

Method.—"With a pipette transfer 25 c.c. of urine into a small Erlenmeyer flask (capacity 200 c.c.). Add one or, at most, two drops of phenolphthalein and 15 to 20 gm. powdered potassium oxalate. Shake about one minute and titrate *at once* with tenth normal hydrate until a faint, yet distinct, pink coloration is produced throughout the contents of the flask. Shaking should be continued during the titration, so as to keep the solution as strong as possible in oxalate." The number of cubic centimeters of sodium hydrate used multiplied by 4 gives the acidity per cent in terms of tenth normal alkali.

The inaccuracy of direct titration of the urine with sodium hydrate, as proposed by Naegeli, is pointed out by Folin. The two chief sources of error are ammonium salts and the occurrence of calcium in the presence of acid phosphates. By first treating the urine with potassium oxalate each of these sources of error is practically eliminated.

Normal values with this method are 25 to 30 acidity per cent (Wood).

SPECIFIC GRAVITY

As a rule, the determination of the specific gravity of the urine is of real value only in the twenty-four-hour specimen. It is usually determined by means of an urinometer. The short, small instruments designed for the purpose of taking the specific gravity of small quantities of urine are usually very inaccurate.

In using the urinometer the urine is carefully poured into a glass cylinder, so that no foam is produced. Should foam collect despite the precautions, even though there be only a few bubbles, they should be removed with filter paper. The cylinder must be sufficiently wide to permit the urinometer to float freely without coming in contact with its wall. The reading is made with the eye on a level with the *bottom* of the meniscus (the concave upper surface of the fluid). The instru-

[7] Folin, O. "The acidity of the urine." *Amer. Jour. Physiol.*, 1903, IX, 265.

[8] A normal solution of acid or alkali should be purchased from a reliable firm. With this as a standard, the physician may easily prepare most of the remaining normal solutions required in routine work.

ments are standardized for use at a temperature of 15° C. ordinarily. For each 3° C. above this temperature the specific gravity is depressed one point in the third decimal place. As an example, if the specific gravity of a urine were found to be 1.015 at 24° C., the corrected reading would be $1.015 + 0.003 = 1.018$.[9]

In case the specimen of urine furnished for examination be small, the urine which remains after the necessary tests have been performed may be diluted with water and the specific gravity of the diluted specimen determined. The last two figures of the specific gravity found are multiplied by the dilution; the result approximates the specific gravity of the undiluted urine.

Normally the specific gravity of the twenty-four-hour specimen varies between about 1.010 and 1.025; absolute limits for the normal cannot be assigned, for so many factors enter into the determination of the specific gravity that in individual instances the figures given may be passed in either direction, without necessarily signifying disease. It must be remembered that readings made with the urinometer are not absolutely correct, but are sufficiently accurate for clinical purposes. Where greater accuracy is required a pycnometer should be employed.

An approximate idea of the amount of solids dissolved in the urine may be obtained by multiplying the last two figures of the specific gravity by 2.33 (Häser's coefficient), the result being the amount of solids in grams.

UREA

The *normal* amount of urea excreted daily in the urine varies within rather wide limits. Values between 20 and 40 gm. are usually found. In clinical work urea determinations have been practically abandoned, except in the diagnosis of unilateral renal disease. And here the determinations are not so extensively employed as formerly. When there is marked damage to one kidney or in cases where one kidney is much more injured by disease than the other, the healthier kidney excretes a greater amount of urea. The urines must, of course, be collected from the kidneys by ureteral catheterization.

Hüfner's Hypobromite Method.—The most convenient apparatus for applying this test is Heinz's modification of the Doremus tube. It con-

[9] In routine examinations, no corrections for temperature are made.

sists of a J-shaped tube mounted on a stand. A bulb is blown in the extreme end of the "tail" of the J-tube and a second tube of 2 c.c. capacity, graduated in $\frac{1}{10}$ c.c., is blown into the upright arm of the J-shaped tube. The connection between the two tubes may be cut by means of a glass cock in the 2 c.c. tube. The upper end of the J-tube is sealed.

The reagent, Rice's bromin solution, is prepared as follows:

Sol. 1.	Sodium hydrate	40.0 gm.
	Distilled water	100.0 c.c.
Sol. 2.	Bromin	10.0 c.c.
	Potassium bromid	10.0 gm.
	Distilled water	80.0 c.c.

The two solutions are kept in separate bottles, and at the time of performing the test, are mixed in equal volumes.

Method.—Fill the small tube with the urine. The stop-cock is then opened until the urine reaches the zero mark. The excess of urine, which has run into the large J-tube, is removed from the latter by washing it with water, the upper end of the tube containing the urine being appropriately sealed to prevent its escape. The J-tube is now filled with the mixed solutions, sufficient of the latter being employed to completely fill the upright (all air must be displaced). The stop-cock is opened and the urine is *slowly* run into the mixed solutions. As the two fluids come in contact, the hypobromite liberates nitrogen gas, which collects at the upper end of the large tube. The volume of gas liberated by 1 c.c. of urine is read on the scale marked on the upright arm of the J-tube, and gives the urea in grams in 1 c.c. of urine.

The method is very inaccurate as a means for determination of urea; the results obtained approach more nearly the total nitrogen of the urine. For this reason the method is inapplicable, where exact values for urea are required, as, for example, in metabolism experiments. In the diagnosis of surgical affections of the kidney, where the urine from each kidney is examined separately, marked differences in the two kidneys are shown with sufficient accuracy, and it is in this connection that the method is used most at the present time.

URIC ACID

The *normal* quantity of uric acid in the urine in twenty-four hours lies between 0.1 and 1.25 gm., with a patient on a mixed diet. The endogenous uric acid of the urine varies between 0.1 and 0.4 gm.

Qualitative Determination of Uric Acid

Qualitative determination of uric acid is made by the *murexid test.* A small drop of the urinary sediment or other material to be tested is dissolved in two or three drops of nitric acid in a porcelain evaporating dish. The material is evaporated to dryness, preferably on a water bath, care being exercised to avoid burning the preparation. The stain which remains on the dish has a reddish color. (A yellow stain may indicate that an insufficient quantity of nitric acid was used.) The addition of ammonium hydrate or, better still, exposing the stain to ammonia fumes changes the color to a purplish red, which fades on heating. The reaction is given by uric acid and by its salts. (For further qualitative tests, see urinary sediments.)

Quantitative Determination of Uric Acid

Quantitative determinations of uric acid are without value unless the patient is on a purin-free diet (eggs, milk, cheese); under these circumstances, the uric acid voided in the urine is entirely endogenous uric acid. The entire twenty-four-hour output of urine must be collected. Determinations are, however, of so little diagnostic value that they have been practically given up in clinical work. Estimations of uric acid in the blood (q. v.) are of greater value and are more easily carried out.

The *endogenous uric acid* excretion of the urine is *increased,* where there is:

1. *Increased proteid catabolism*—usually those conditions in which total nitrogen is increased, as in fevers, in all diseases where there is rapid emaciation, as in hyperthyroidism.
2. Also in *leukemia* (up to 8 gm. Magnus-Levy).
3. In *pneumonia,* during the resolution of the exudate.
4. In *hepatic cirrhosis* at times.
5. After administration of *atophan.*

There is a *decreased* excretion:

1. In *nephritis.*
2. In *gout* between the acute attacks, rising to normal during the attacks.
3. After large doses of *quinin.*
4. In some *chronic diseases.*
5. By a *poor diet.*

The formation of uric acid crystals in the urine is not indicative of gout. It *may* indicate a uric acid diathesis, *so-called,* in that it is very often associated with uric acid calculi in the pelvis of the kidney or in the urinary bladder. However, uric acid may be precipitated in crystalline form in *any* very acid urine.

Method of Folin and Shaffer.[10]

Reagents:

Sol. 1.	Ammonium sulphate	500.0 gm.
	Uranium acetate	5.0 gm.
	Distilled water to	650.0 c.c.
	Dissolve and then add:	
	Acetic acid, 10 per cent	60.0 c.c.
	Distilled water to	1,000.0 c.c.
Sol. 2.	Ammonium sulphate	100.0 gm.
	Distilled water to	1,000.0 c.c.
Sol. 3.	$\frac{N}{20}$ potassium permanganate.	

To prepare the twentieth normal permanganate solution, dissolve 1.7 gm. of potassium permanganate in one liter of distilled water. The solution is boiled or autoclaved to render it more permanent. After it has cooled to room temperature, it is titrated against tenth normal oxalic acid solution (6.3 gm. purs crystals to one liter of distilled water). With a pipette, 10 c.c. of $\frac{N}{10}$ oxalic acid are placed in a small Erlenmeyer flask or beaker, diluted with about 100 c.c. of distilled water, and 15 c.c. of concentrated sulphuric acid added. The temperature of the mixture is raised, by the addition of the sulphuric acid, to about 60° C. While

[10] Folin, O., and Shaffer, P. A. "Ueber die quantitative Bestimmung der Harnsäure im Harn." *Ztschr. f. physiol. Chem.,* 1901, XXXII, 552.

still hot the permanganate solution is added to it from a burette, under constant stirring, until a uniform red color appears, which persists throughout the fluid for a few seconds.[11] This is the end reaction. The quantity of permanganate solution used is read off on the burette. Since the permanganate solution has been made too strong, less than 20 c.c. of it should have been required to produce the end reaction. The permanganate solution which remains is accurately measured (with volumetric flasks and pipettes, not with cylinders), and is diluted with distilled water, so that exactly 20 c.c. will give the end reaction with 10 c.c. of tenth normal oxalic acid. When kept in a dark place, tightly stoppered, the potassium permanganate solution is fairly permanent for several months. The titer should, however, be determined from time to time with the oxalic acid solution.

Example.—If 18.9 c.c. of permanganate solution gives the end reaction with 10 c.c. of $\frac{N}{10}$ oxalic acid, and the remainder of the potassium permanganate solution amounts to 960 c.c., the necessary dilution to make the permanganate solution twentieth normal is determined by the following equation: $18.9 : 20 :: 960 : x$. $x = 1{,}015.8$. Therefore, the amount of water necessary to add would be $1{,}015.8 - 960$ or 55.8 c.c.

Method.—To 300 c.c. of urine in an Erlenmeyer flask or beaker of 500 c.c. capacity or larger, add 75 c.c. of the uranium acetate reagent (sol. 1) to precipitate phosphates and other substances, which might interfere with the accuracy of the method. Both urine and reagent must be accurately measured with volumetric pipettes. Stir the mixture well, and allow it to stand five minutes. Then filter through a double folded filter. Measure with a pipette 125 c.c. of the filtrate (this represents 100 c.c. of the urine originally used) into each of two beakers and add 5 c.c. of ammonium hydrate to each to convert the uric acid into ammonium urate. Mix well, and set aside for twenty-four hours; by the end of this time the precipitate will have settled to the bottom of the beaker. The clear, supernatant fluid is decanted, the precipitate collected on a filter (Schleicher and Schüll's No. 597) and washed with 10 per cent ammonium sulphate, till the filtrate is almost chlorin free. (In testing for chlorids add a little nitric acid and then a few drops of dilute silver nitrate solution [10 to 15 per cent solution]; a white

[11] Early in the titration, after the addition of the first few drops of permanganate, a red color, which may persist for fifteen seconds, or so, may be noted, but it quickly disappears on adding more permanganate.

precipitate or cloud is formed if chlorids are present.) The filter paper is now pierced with a glass rod, and the precipitate washed into a beaker with about 100 c.c. of distilled water. Add 15 c.c. of concentrated sulphuric acid and titrate the mixture immediately, stirring constantly, until a pink color appears throughout the fluid and persists for a few seconds.

Each cubic centimeter of twentieth normal permanganate is equivalent to 3.75 mg. of uric acid. This, multiplied by the number of c.c. of permanganate used, gives the uric acid in 100 c.c. of urine, from which the total amount for twenty-four hours is readily calculated. Since ammonium urate is slightly soluble in water and somewhat more so in urine, a correction of 3 mg. should be added to the final result for each 100 c.c. of urine.

AMMONIA

Normaliy the urine contains 0.6 to 0.8 gm. of ammonia daily for the average adult on a mixed diet. The limits of the normal are about 0.3 to 1.2 gm. In health the ammonia nitrogen usually amounts to 4 to 5 per cent of the total nitrogen, when a mixed diet is taken.

In clinical work, determinations of the ammonia elimninated in the urine are carried out much less frequently now than formerly. It may be useful at times in following the degree of acidosis, since the acids formed in the body are neutralized largely by ammonia, thus sparing the fixed alkalies. Therefore, at the height of an acidosis, the excretion of ammonia is high, falling as the degree of acidosis diminishes.

In the list of conditions in which a high ammonia excretion has been noted, it will be seen that acidosis [12] is the most important factor.

The *ammonia* of the urine is *increased* in:

1. *Acidoses,* as, for example, in *diabetic acidosis.*
2. *Mineral acid poisoning.*
3. Normal individuals on a *rich protein diet.*
4. *Fevers.*
5. *Cirrhosis of the liver.*
6. *Starvation.*
7. *Pernicious vomiting* of pregnancy.
8. *Acute yellow atrophy* of the liver.

[12] The word *acidosis* is used in its broadest sense, not referring to any special type of acidosis.

9. *Phosphorus poisoning.*
10. *Cancer* at times.
11. Many *gastro-intestinal diseases,* especially in children.
12. *Ether* narcosis.

Quantitative Determination of Ammonia

Folin's Method.[13]

Reagents:
Sodium chlorid.
Anhydrous sodium carbonate.
Petroleum or toluene.
$\frac{N}{10}$ sulphuric acid; $\frac{N}{10}$ sodium hydrate.
One per cent aqueous solution of alizarin red.

The apparatus required includes aërometer cylinders (45 cm. deep and 5 cm. in diameter), a suction pump, calcium chlorid tube, doubly perforated stoppers to fit the cylinders, and tubing for connections. Folin's tube to secure thorough mixing of air and acid is a convenience, though not a necessity. The apparatus is connected as shown in the illustration (Fig. 1), the calcium chlorid tube filled with cotton being placed between the cylinders to prevent the alkaline urine being drawn over into the acid.

Method.—With a pipette, 25 c.c. of tenth normal acid are placed in cylinder *B* and diluted with distilled water sufficiently to cover the end of the mixing tube. Into cylinder *A,* 25 c.c. of the twenty-four-hour specimen of urine are measured with a pipette. To the urine are added 8 to 10 gm. of sodium chlorid, 5 to 10 c.c. of toluol or petroleum to prevent foaming (with blood or other fluid rich in protein add some methyl alcohol also), and, finally, about 1 gm. of anhydrous sodium carbonate. After the addition of the soda the cylinder is immediately stoppered and the air current started. Before entering the urine the air current may be passed through a wash bottle containing sulphuric acid to remove ammonia in the air, though usually this precaution is unnecessary.

The addition of the soda to the urine liberates the weaker base,

[13] Folin, O. "Eine neue Methode zur Bestimmung des Ammoniaks im Harne und anderen thierischen Flüssigkeiten." *Ztschr. f. physiol. Chem.,* 1902-03, XXXVII, 161.

ammonia, which is carried over by the air stream into the tenth normal sulphuric acid, by which it is neutralized. When Folin's mixing tube is used, not a trace of the ammonia escapes neutralization by the acid, even though there remains an excess of only 5 c.c. of tenth normal acid. If ordinary glass tubing be employed to pass the air through the acid, a second cylinder, containing 10 c.c. of tenth normal acid, should be interposed between the acid cylinder and the pump to catch the ammonia which escapes neutralization.

The air pump should be capable of carrying 600 to 700 liters of

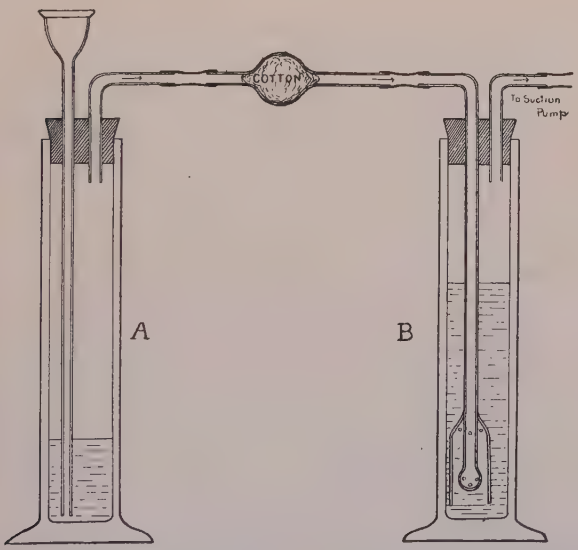

FIG. 1.—APPARATUS FOR THE QUANTITATIVE DETERMINATION OF AMMONIA ACCORDING TO FOLIN. Cylinder *A* for urine, Cylinder *B* for acid.

air per hour through the apparatus. With a pump of this capacity working at room temperature (20 to 25° C.) all of the ammonia is carried into the acid in an hour or an hour and a half.[14]

When the process is completed the acid is poured into an Erlenmeyer flask or beaker and the cylinder rinsed with distilled water, which is

[14] The efficiency of the pump and its "working time" are readily tested by taking a specimen of urine and titrating the acid at the end of an hour; add more tenth normal acid and titrate at fifteen-minute intervals, until acid is no longer neutralized.

added to the acid. The acid is now titrated with tenth normal sodium hydrate, using two drops of alizarin red to 200 to 300 c.c. of fluid. The end reaction is the appearance of a red color throughout the fluid; do *not* continue the titration to the appearance of a violet color. The difference between the number of cubic centimeters of acid originally taken and that of the alkali used is the number of cubic centimeters of acid neutralized by ammonia. Since one cubic centimeter of tenth normal acid is equivalent to 0.0017 gm. of ammonia, this, multiplied by the number of c.c. of acid neutralized, gives the quantity of ammonia in 25 c.c. of urine. The quantity in the twenty-four-hour specimen is calculated from this.

Determinations may be made in duplicate or triplicate by connecting two or more sets of apparatus in series.

The Vacuum Distillation Method.—Shaffer [15] has modified the vacuum

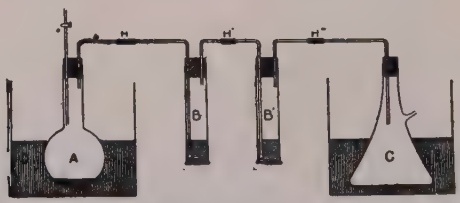

Fig. 2.—Apparatus for the Determination of Ammonia According to Shaffer (after Shaffer).

distillation method. As described by him, it is carried out in the following manner: To 50 c.c. of urine in flask *A* (Fig. 2) add an excess (15 or 20 gm.) of sodium chlorid, and about 50 c.c. methyl alcohol. In bottle *B* place 25 or 50 c.c. $\frac{N}{10}$ acid and in *B'* 10 c.c. $\frac{N}{10}$ acid, diluted in each case with a small amount of water. If too much water is added there will be danger of loss of acid by jumping over during the violent commotion which is set up in the acid by the rapid passage of the steam. If such a loss should occur the acid can always be recovered by rinsing out the filter flask *C*. When the apparatus is ready, about 1 gram dry sodium carbonate is added to the liquid in flask *A*, the stopper quickly put in place, and the suction started. With a good pump the pressure will be reduced to about 10 mm. Hg. in two or three minutes, when, the liquid surrounding *A* being at 50° C., a rapid boiling will begin.

[15] Shaffer, P. "On the quantitative determination of ammonia in urine." *Amer. Jour. Physiol.*, 1903, VIII, 330.

The temperature is maintained, and the boiling allowed to continue for fifteen minutes. At the end of that time the ammonia will in all cases have been completely given off, and the operation may be stopped by slowly letting in air at the stopcock in tube *a*. The acid in *B* and *B'* is titrated and the ammonia calculated. One per cent aqueous alizarin red is used as the indicator. (For a description of the end-point and the calculation see the preceding method of Folin.)

This method is very accurate, and consumes very little time. Shaffer found the results in all cases correct within less than 10 mg. of ammonia per liter. Where the necessary apparatus is available, this method or that of Folin is to be preferred.

NITROGEN

The total nitrogen of the urine of a *normal adult* on a mixed diet lies usually between 10 and 16 gm., or about 0.2 gm. per kilo of body weight.

Total nitrogen output in the urine is greater than the intake:

1. Rich *protein diet.*
2. Diseases accompanied by marked *malnutrition* or *starvation.*
3. *Acute infections.*
4. *Hyperthyroidism.*
5. *Carcinoma and sarcoma.*
6. *Chronic infections,* such as tuberculosis.
7. *Leukemias.*
8. *Phosphorus poisoning.*
9. *Scurvy.*
10. During *absorption of exudates.*
11. *Nephritis* at times (albumin of the urine).
12. *Diabetes mellitus* at times.

Nitrogen output is *diminished:*

1. *Convalescents.*
2. At times in the later stages of *Bright's disease.*

Very little information of diagnostic value is gained by estimating the total nitrogen of the urine alone. The patient must be on a fixed diet, so that the intake of nitrogen may be known.

Kjeldahl's Method for Determination of Total Nitrogen.

Reagents:[16]

Crystalline copper sulphate.
Crystalline potassium sulphate.
Concentrated sulphuric acid.
40 per cent solution of sodium hydrate.
Talc powder.
$\frac{N}{10}$ sulphuric acid; $\frac{N}{10}$ sodium hydrate.
One per cent aqueous solution of alizarin red, or tincture of cochineal.

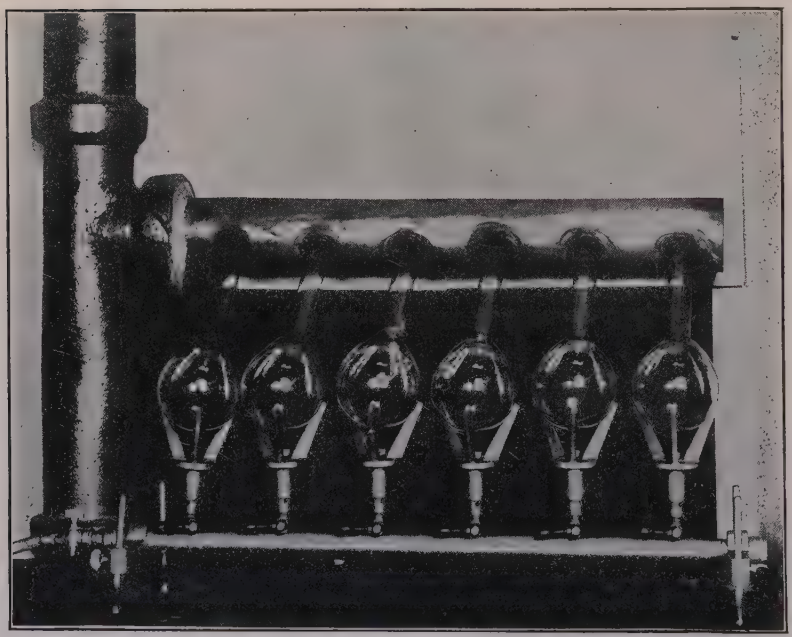

FIG. 3.—DIGESTING RACK FOR THE KJELDAHL NITROGEN DETERMINATION. The necks of the flasks extend into a perforated lead pipe, which is connected with a tile outlet.

Method.—With a pipette 5 c.c. of the twenty-four-hour specimen of urine are measured into a Kjeldahl oxidizing flask (Jena glass) of about 800 c.c. capacity. Then add about 15 c.c. of concentrated sul-

[16] To determine whether the reagents are N-free, proceed with the method, substituting 5 c.c. of glucose solution for the urine. If nitrogen is found, the necessary correction is evident.

phuric acid and about 0.2 gm. of copper sulphate crystals, and, finally, about 10 gm. of potassium sulphate. The flask is placed under a hood [17] and is heated over a Bunsen burner,[18] with a low flame at first, until the foaming has ceased. The heating is continued till the contents of the flask become clear. It may be necessary to remove the flask, so that all the charred matter may be brought into the acid. After the fluid in the flask has become pale green or colorless, the heating is prolonged fifteen minutes to insure complete oxidation. All of the nitrogenous compounds have been converted to ammonia, which unites with the sulphuric acid to form ammonium sulphate. The liquid is allowed to cool (it may crystallize eventually); about 300 c.c. of distilled water are then added, and, when solution is obtained, a heaping teaspoonful of talc powder is placed in the flask (to prevent bumping during the boiling). Finally, sufficient 40 per cent sodium hydrate is added to render the solution strongly alkaline; the quantity required must have been determined previously. It is well to incline the flask and pour the alkali down the side, to prevent mixing and possible loss of ammonia.[19] The flask is immediately connected with a distilling apparatus (Fig. 4) provided with a Hopkins bulb or similar device to prevent alkali passing over into the acid, and its contents boiled. The distillate, containing the ammonia liberated by the stronger alkali, is received in an Erlenmeyer flask in which 25 to 50 c.c. of tenth normal sulphuric acid have been placed. The distillation is continued till the distillate is no longer alkaline to litmus—usually a half hour or less. The condensing tube is then washed with distilled water into the distillate. To determine the excess of acid, the contents of the flask are titrated with tenth normal alkali with alizarin red (2 drops to 200 to 300 c.c. of fluid) as the indicator. The end-point is a red color, not a violet. (Tincture of cochineal [20] is used at times as an indicator. It

[17] A lead pipe, perforated with holes to receive the necks of the digesting flasks (see Fig. 3) and connecting with a flue, is better than most hoods. If there is a good draught, the fumes are carried off perfectly. An outlet constructed of tile pipes is inexpensive and satisfactory.

[18] The most satisfactory form of apparatus is that designed by Folin and made by the International Instrument Co., Cambridge, Mass. The small model is shown in Fig. 3.

[19] To guard against such loss, a doubly perforated stopper may be provided and the alkali introduced into the flask by a funnel, whose stem, passing through the stopper, is plugged immediately after adding the alkali.

[20] Tincture of cochineal is prepared by grinding cochineal bugs in 50 per cent alcohol in a mortar, allowing the mixture to digest a day, and filtering.

imparts a very pale brown color to acid solutions when added in the proportion of about four drops to 300 c.c.; when the reaction becomes alkaline the color changes to amethyst.) Since one c.c. of tenth normal acid is equivalent to 0.0014 gm. of nitrogen, the amount of the latter in 5 c.c. of urine and, finally, in the twenty-four-hour specimen is easily computed.

If insufficient acid has been taken to receive the distillate, the excess

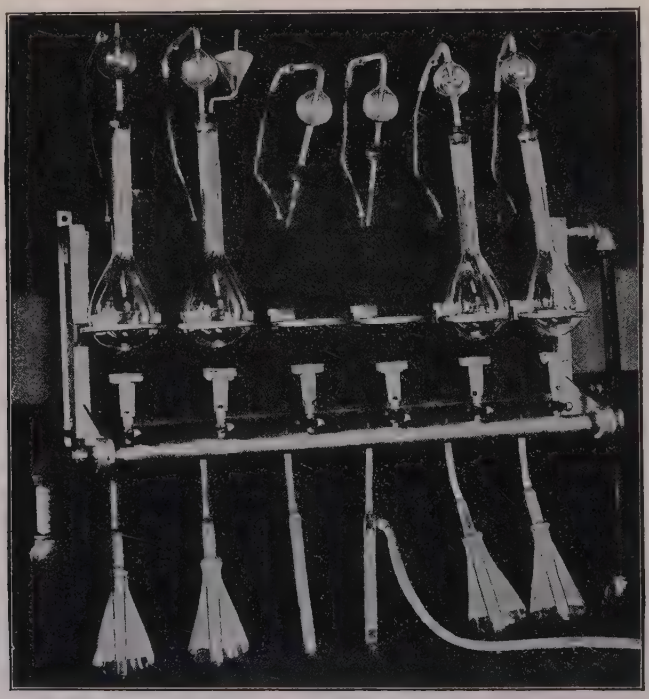

Fig. 4.—Distilling Apparatus for the Kjeldahl Nitrogen Determination.

of ammonia may be titrated with tenth normal sulphuric acid (Hoppe-Seyler, Thierfelder).

Instead of distilling the oxidized material, the ammonia determination may be performed according to Folin's method. The titration and calculation of the result are performed as described above.

CHLORIDS

In health, with the usual mixed diet, the chlorids of the urine usually amount to 10 to 15 gm. daily. The limits of the normal are said to be 6 and 22 gm.

Chlorids in the urine are *reduced* in:

1. Febrile diseases, especially those in which an exudate is formed, as in *lobar pneumonia, pleurisy with effusion, meningitis.*
2. *Severe diarrheas.*
3. Many cases of *nephritis,* especially parenchymatous nephritis, and in *circulatory failure* with edema.
4. Persistent *vomiting.*
5. *Diminished absorption,* as in carcinoma of the pylorus.
6. *Chronic wasting diseases* (inconstant).

Chlorids may be increased:

1. After *chloroform anesthesia.*
2. In *diabetes insipidus.*

The value of chlorid determinations in the urine is limited. In central pneumonia, where physical signs are lacking or doubtful, a great decrease in the chlorids affords corroborative evidence of some value. The qualitative test usually suffices for this purpose, a known normal urine being used as a control.

Qualitative Test

About 10 c.c. of urine, placed in a test tube, are acidified with strong nitric acid, and then one or two drops of dilute silver nitrate solution (10 to 15 per cent aqueous solution) are added. A white precipitate denotes the presence of chlorids. An approximate idea of the quantity of chlorids may be gained. Normally, a dense precipitate appears and quickly settles to the bottom of the tube. With great reduction in the chlorids only a cloud is seen, without flocculent precipitate.

Quantitative Determination of Chlorids

Harvey's [21] Modification of Volhard's Method.—This method is valuable because of its rapidity without sacrifice of accuracy. As in the

[21] Harvey, S. C. "The quantitative determination of the chlorids in the urine." *Arch. Int. Med.,* 1910, VI, 12.

original method, the chlorids are precipitated by adding an excess of silver nitrate, the amount of which is determined by titration with ammonium thiocyanate. The chlorids are calculated as sodium chlorid. Albumin, if present in the urine in appreciable quantity, must be removed by boiling and the subsequent addition of dilute acetic acid, before proceeding to the estimation of the chlorids. Largely in the author's words, the method follows:

Reagents:

a. A silver nitrate solution containing 29.042 gm. of chemically pure, crystalline silver nitrate in one liter of distilled water; 1 c.c. of this solution is equivalent to 0.01 gm. of sodium chlorid. (The silver solution may be standardized against a weighed quantity of dry, chemically pure sodium chlorid.)

b. A solution of ammonium thiocyanate, 20 c.c. of which is equivalent to 10 c.c. of the silver nitrate solution. As this salt is very hygroscopic, it cannot be weighed with sufficient accuracy to make the solution directly. Therefore, 13 gm. of it are dissolved in one liter of distilled water, thus making a concentrated solution, whose strength is determined by titration against the silver nitrate solution, and the requisite dilution made. This is done in the following manner: 10 c.c. of the silver nitrate solution are measured with a pipette into a beaker, diluted with about 20 c.c. of distilled water, 2 c.c. of the indicator (sol. *c*) added, and the whole titrated with the ammonium thiocyanate solution. If, for example, 12 c.c. of the solution are used in the titration and the total volume of the thiocyanate solution is 960 c.c., the volume to which it must be diluted with distilled water is determined according to the equation $12:20::960:x$, in which x represents the required volume.

c. The *indicator* containing nitric acid. To 30 c.c. of distilled water add 70 c.c. of nitric acid (sp. gr. 1.2, or 33 per cent). Saturate this menstruum with crystalline ferric ammonium sulphate and filter.

This indicator is recommended, inasmuch as it substitutes one solution in place of the two (the ferric indicator and the acid), and insures the use of the proper amount of the acid. Moreover, it is sufficiently concentrated, so that it is necessary to use only 2 c.c., and, therefore, it may be kept in a small reagent bottle. The stopper of this bottle may be a graduated dropper, which can at the same time serve to measure and transfer the indicator.

Method.—With a pipette transfer 5 c.c. of the twenty-four-hour

specimen of urine (albumin-free) to a small beaker or Erlenmeyer flask, and dilute it with about 20 c.c. of distilled water.[22] The chlorids in this solution are now precipitated by adding 10 c.c. of the silver nitrate solution with a pipette. Next, place about 2 c.c. of the indicator in the mixture. The ammonium thiocyanate solution is then run in from a burette under constant stirring, until the first trace of red shows throughout the mixture. On allowing the precipitate to settle, the color may easily be recognized in the supernatant fluid. If, however, the mixture is stirred violently, the color will disappear. When the end-point appears on the addition of the first drop of ammonium thiocyanate solution (i. e., when the original 10 c.c. of silver solution is insufficient to precipitate all the chlorid), then 10 c.c. more of the silver nitrate solution are added, and the titration completed with corresponding allowance in the calculation.

The calculation may be made as follows: As 20 c.c. of the ammonium thiocyanate solution are equivalent to 10 c.c. of the silver nitrate solution, divide the number of c.c. of thiocyanate solution used by two (2) and subtract the quotient from 10 c.c., the amount of silver nitrate originally taken. The result is the number of c.c. of silver nitrate solution actually used in the precipitation of the chlorids. As 1 c.c. of the silver solution is equivalent to 0.01 gm. of sodium chlorid, the number of cubic centimeters of silver nitrate solution used, multiplied by 0.01, will give the amount of the chlorids, expressed in terms of sodium chlorid, in 5 c.c. of urine, the quantity taken. From this the total amount of chlorid in the twenty-four-hour specimen is calculated.

INDICAN

Quantitative estimation of the sulphates in the urine is of no practical value in general clinical work at the present time. Of the sulphates present in the urine, indoxyl sulphate alone is tested for in the usual examination.

The information gained in testing the urine for indican is of so little value, in the experience of the writer, that it should not form a part of the routine examination. It is a decomposition product of

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[22] When the urine is highly colored, add 8 to 10 per cent solution of potassium permanganate a drop at a time, until the red color no longer fades rapidly, and the urine has become pale yellow.

protein, and an increased elimination may be found, therefore, whenever there is an increased destruction of protein, either in the intestinal tract, as in many cases of chronic constipation or after a rich protein diet, or in other parts of the body, as in cases of pulmonary gangrene, or, often, in empyema. From a diagnostic standpoint, the information gained through testing for indican is not worth the time spent in performing the test. There has been much loose talking and writing regarding indicanuria.

The tests for indoxyl sulphate depend on the oxidation of indoxyl to indigo blue and its extraction in chloroform. It is necessary at times to precipitate the urine, before testing, with one-fifth volume of 20 per cent lead acetate to remove pigments, which may interfere with the recognition of the blue color.

Obermayer's Test.—Equal parts of urine and Obermayer's reagent (0.2 per cent ferric chlorid in concentrated hydrochloric acid) are mixed in a test tube and allowed to stand a few minutes (2-3). A small amount of chloroform is added, and the test tube is inverted several times. With normal amounts of indoxyl sulphate a faint blue is seen in the chloroform; an excess causes a dark blue color. By using the same quantities of urine, reagent, and chloroform, and test tubes of uniform diameter, daily variations in the intensity of the reaction may be followed.

Jaffé's Test.—Equal quantities of urine and strong hydrochloric acid are mixed in a test tube; about 2 c.c. of chloroform and 1 to 3 drops of strong aqueous solution of calcium hypochlorite are added. The tube is inverted several times, and the indigo collects in the chloroform, as in the preceding test.

If the patient has been receiving *iodin* in any form, a violet color is imparted to the chloroform in performing Obermayer's and Jaffé's tests. To destroy the color produced by the iodin and bring out that of indigo blue, if present, the chloroform is transferred to a second test tube and is shaken with dilute potassium hydroxid; or water and a small quantity of strong sodium thiosulphate solution are added to the chloroform and the whole shaken. The violet is decolorized, leaving the blue.

Codein, when administered in large doses, is said to give a purplish red color to the chloroform.

ALBUMIN

Normal urine contains albumin in traces too small to be detected with the usual tests.

Albumin occurs in the urine in *normal individuals:*

1. In the so-called *physiological albuminuria.* The quantity of albumin is usually greater when the patient is in the erect posture. In *orthostatic albuminuria,* the excretion of albumin occurs only when the patient is in the erect posture; in *hypostatic albuminuria,* on the other hand, albumin is excreted only when the patient is recumbent.
2. After *severe physical exertion,* as, for example, in athletes after a Marathon race, a foot-ball game, etc.
3. During *labor* (in about 50 per cent of cases).
4. From *traumatism,* i. e., palpation of the kidneys (in 15 of 21 cases reported by Menge).
5. At *puberty* in certain instances.
6. In *new-born infants* during the first 8 to 10 days of life. In this instance, hyaline casts are usually found; they are absent, as a rule, in the preceding types.
7. *Alimentary albuminuria,* after the consumption of large quantities of protein; for example, 4 to 10 raw eggs.

In the albuminurias occurring in healthy subjects (enumerated above), *nucleo-albumin* is usually present with albumin, while in nephritides it is missed as a rule (Krehl).

Albuminuria is met with *in disease:*

1. In *nephritis.*
2. In *circulatory disturbances,* either general or local, which interfere with the circulation of the blood in the kidneys.
3. In *toxemias,* such as occur in acute infectious fevers, jaundice, etc.
4. *Poisons* introduced into the body from without, such as bichlorid of mercury, turpentine, etc.
5. After long continued exposure to *cold.*
6. After *extensive burns of the skin.*
7. *Extrarenal lesions.* Albumin may appear in the urine as a component of an inflammatory exudate, from rupture of an abscess

into the genito-urinary passages, from hemorrhage, etc. In the case of extra-renal albuminurias, casts are lacking, unless, of course, there is a co-existing renal lesion.

Before testing for albumin, two conditions must be fulfilled: (1) The urine must be perfectly clear, and (2) its reaction must be acid.

1. If the specimen to be examined is fresh and fairly clear, passage through filter paper usually suffices to render it transparent and clear. With urines containing abundant fine precipitates or many bacteria, simple filtration is not satisfactory. Such urine should be shaken with Kieselguhr (infusorial earth) and then passed through a folded filter. The meshes of the paper are plugged, so that the filtrate is perfectly clear, though it may be necessary to return the first few cubic centimeters of the filtrate to the filter. Minute quantities of albumin may be removed by the filtration with Kieselguhr.

2. If alkaline or neutral in reaction, the urine should be rendered slightly acid to litmus by the addition of a few drops of 3 per cent acetic acid.

Of the following qualitative tests it is advisable to use at least two in all instances to avoid error. Heller's and the heat and acetic acid tests form a satisfactory combination.

Qualitative Tests

1. **Heat and Acetic Acid Test.**—(*a*) First Method.—A test tube (18 to 20 mm. in diam.) is nearly filled with the clear, acid urine. Holding the tube by its lower end, the urine in the upper part is boiled over a Bunsen burner or spirit lamp, the cool urine in the lower part of the tube serving for comparison with the boiled portion. A cloud may appear on boiling, due (1) to precipitation of calcium phosphate, or (2) to albumin, or (3) to the precipitation of both simultaneously. A few drops of 3 per cent acetic acid are now added. If the precipitate be due to phosphates alone, it will disappear on the addition of the acid, whereas the albumin coagulum will usually be intensified, never lessened, unless a considerable excess of acid is added. When both phosphates and albumin are precipitated together, the cloud may be perceptibly diminished but not abolished by acidification. Very small quantities of albumin may give no cloud on heating, but the albumin may appear after the addition of the acid. Such traces of albumin are best detected by holding the tube against a dark background with

the eye at a right angle to the source of light, for the faint cloud may be easily overlooked on casual inspection. The urine in the upper part of the tube (which has been boiled) is compared with the clear urine in the lower part of the test tube. When the urine is of very low specific gravity and, therefore, poor in salts, the test is improved by the addition of one-fifth to one-tenth volume of saturated sodium chlorid solution to the urine. The urine is not to be boiled after the addition of the acid.

The test is said to indicate albumin in a dilution of 1:130,000 (Glaesgen).

Sources of Error.—(*a*) There is danger in adding too much acetic acid, since the albumin may be converted into the soluble acid albumin or syntonin. That it requires a considerable excess of acid to redissolve the precipitate, however, once it is formed, is easily demonstrated. It is helpful to the worker to experiment with known specimens to determine the degree of latitude one can safely follow in the addition of the acid. (*b*) Nucleo-albumin may be precipitated by heat and acetic acid; it is also thrown out of solution by the addition of dilute acetic acid to the *cold* urine. Two tests may be performed, one on the cold, the other on the boiled urine; by comparison it is usually possible to estimate whether part or all of the precipitate is due to the nucleoproteid. Or, the urine is treated with dilute acetic acid, filtered to remove the precipitate of nucleo-albumin, a few more drops of the dilute acid added, and the contents of the test tube boiled; a precipitate appearing now is albumin. The test for nucleo-albumin is improved if the urine be diluted with water; that for albumin is sharper after the addition of salt. (*c*) Following the administration of cubebs, copaiba, turpentine, etc., resinous bodies appear in the urine, and may be precipitated. After cooling the fluid the precipitate may be dissolved in petroleum benzine or in alcohol, albumin being insoluble. (*d*) Albumoses appear after the urine becomes cool; the precipitate redissolves on boiling. (*e*) The Bence-Jones body is coagulated at about 60° C., but usually redissolves in part or wholly as the boiling point is reached. (*f*) Bile salts are precipitated by dilute acetic acid added to the *cold* urine. It has been found that this reaction is obtained with bile salts in dilutions as high as 1:6,400.[23] The salts are also precipitated in the boiled urine.

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[23] Oliver, S. F. "The effect of bile salts in the urine on routine tests for albumin." *Jour. Lab. and Clin. Med.*, 1922, VII, 743.

(*b*) Second Method.—This method, widely used in France, has recently been carefully examined and recommended by Glaesgen.[24] The acetic acid is added before the specimen is boiled. About 20 c.c. of urine and 5 drops of 20 per cent acetic acid are mixed in a test tube. The urine in the upper part of the tube is boiled (or the mixture may be divided between two test tubes, one to be boiled, the other to serve as a control). If the acetic acid produces a cloud in the cold (nucleoproteid or bile salts), the specimen is cleared by filtration before boiling. The acidification previous to boiling prevents a precipitation of phosphates in the majority of instances; if such a precipitate occurs, a few more drops of the acid are added to dissolve it. This will not cause the solution of a slight albuminous precipitate, provided the specimen is not reboiled. With the precautions given, the presence of a cloud or precipitate indicates albumin. (For the detection of a very faint cloud, see the first method.)

By this method Glaesgen finds that albumin may be demonstrated in a dilution of 1:180,000.[25]

2. **Heat and Nitric Acid Test.**—The method of procedure is the same as in the preceding test (first method), the urine in the upper part of the test tube being boiled. One to four drops of concentrated nitric acid[26] are now added. The precipitate, which may form on boiling the urine, may be due to albumin or phosphates or to both. The phosphate precipitate is dissolved by the addition of the acid; in such case a few more drops of nitric acid are added, when albumin is precipitated, if present. When more than a trace of albumin is present in the urine, the precipitate is flocculent and whitish or brownish. With a urine of low specific gravity the addition of one-fifth volume of saturated sodium chlorid solution at times makes it possible to recognize a trace of albumin, which would otherwise be missed. The urine may remain clear after boiling, but a precipitate of albumin may still appear on acidification, as in the heat and acetic acid test. If the cloud is faint, there is danger of missing it, unless the tube be held against a dark background with the eye at a right angle to the source of light. Do not boil after adding the acid.

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[24] Glaesgen. "Zur Methodik des Nachweises sehr kleiner pathologischer Eiweissmengen im Harn." *München. med. Wchnschr.*, 1911, LVIII, 1123.

[25] A somewhat limited experience with the second method has shown it to be quite as sensitive as the first, in the writer's hands.

[26] Nitric acid becomes yellow from the formation in it of nitrous acid. It is readily cleared by the addition of crystals of urea.

The test is said to be as delicate as the heat and dilute acetic acid test.

Sources of Error.—The possibilities of error are much the same as in the heat and acetic acid test. (*a*) An excess of acid is to be avoided, as the precipitate may be dissolved, forming acid albumin. The proportion of acid to urine should not exceed about 1:1,000 (Simon). (*b*) Resinous bodies are distinguished as in the preceding test. (*c*) Uric acid may precipitate after standing a few minutes. The precipitate is crystalline, and gives the murexid test. (*d*) Albumose is soluble in the boiling solution, but insoluble in the cold. The precipitate which forms may be redissolved by heating. (*e*) Bence-Jones protein usually exhibits maximal precipitation at about 60° C., with partial or complete disappearance of the coagulum at the boiling point. (*f*) In markedly icteric urine a green precipitate of biliverdin may be produced. This, unlike coagulated albumin, is soluble in alcohol. Finally, it may be added, the nitric acid possesses an advantage over dilute acetic acid, since its addition to boiling urine does not precipitate mucin or nucleoproteid. The nitric acid must be free from nitrous acid.[27]

3. **Heller's Test.**—In performing this test a wide test tube or, better still, a conical glass or horismascope should be used. Ten to 20 c.c. of urine are placed in a conical glass, and then, with the glass inclined, concentrated nitric acid[28] is poured slowly down its side. Being the denser fluid, the acid collects at the bottom. The glass is now brought to the vertical position very gradually, to prevent mixing of the urine and acid. If albumin is present in the urine, a white precipitate is formed *at* the line of contact between urine and acid. The precipitate is acid albumin, which is insoluble in the great excess of acid. The breadth and sharpness of the ring will depend upon the quantity of albumin present, and also upon the success with which the urine and acid have been layered. When small quantities of albumin are present the ring may appear only after two or three minutes, and then may be overlooked unless the tube is examined against a dark background with the eye at a right angle to the source of light.

Glaesgen[29] finds the reaction positive with albumin in a dilution of 1:35,000.

Sources of Error.—(*a*) Urines which have been preserved with

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[27] This refers to footnote [26] on p. 28, beginning "Nitric Acid."

[28] See footnote [26] on p. 28.

[29] *Loc. cit.*, page 28.

thymol may give a ring at the line of contact which is practically indistinguishable macroscopically from that produced by albumin.[30] Below the ring there is a greenish zone extending into the acid, above it a red zone. When thymol and albumin coexist, it may be noted that the thymol ring forms just beneath that of albumin. The thymol may be removed by shaking the urine with an equal volume of petroleum ether for two or three minutes. (*b*) Urates may be precipitated, but the ring is ½ to 1 cm. *above* the line of contact. The ring is broader than that caused by albumin, and disappears on warming the urine. (*c*) Nucleo-albumin may produce a ring ½ to 1 cm. *above* the line of contact. As nucleo-albumin is insoluble in strong acid, the ring rises as the acid diffuses upward in the urine. The ring is more marked if the urine be diluted with about three parts of water. (*d*) Resinous acids may form a ring above the line of contact. The ring is partially cleared on heating. The precipitate, if due to resins, may be pipetted off and dissolved in ether. When resinous bodies ar suspected the following test may be employed: To 8 to 10 c.c. of urine add 2 to 3 drops of strong hydrochloric acid; the resinous bodies are precipitated. Render strongly acid with hydrochlorid acid and heat; a red color develops. (*e*) Albumose and Bence-Jones body form a ring at the line of contact, which disappears more or less completely on heating. (*f*) Urea nitrate may be deposited between the fluids. It is easily recognized, as it is not compact and uniform, but manifestly crystalline. Dilution of the urine causes its disappearance. (*g*) Bile salts, when present in dilutions as high as 1:6,400, may give rise to a ring just above the line of contact. On standing the ring tends to diffuse upward, so that after about an hour, the whole upper layer (of urine) becomes clouded. The ring is a broad, milky one just above the line of contact of urine and reagent, nearer the line of contact than that produced by nucleo-albumin and urates.[31]

4. **Potassium Ferrocyanid and Acetic Acid Test.**—To 10 to 15 c.c. of urine in a test tube add a few drops (about 5) of strong acetic acid to render the urine markedly acid. Nucleo-albumin, if present, is precipitated and should be removed by filtration. Now add a few drops of 5 per cent potassium ferrocyanid. A cloud or a flocculent precipi-

[30] Weinberger, W. "Thymol as a source of error in Heller's test for urinary protein." *Jour. A. M. A.*, 1909, LII, 1310.

[31] Oliver, S. F. "The effect of bile salts in the urine on routine tests for albumin." *Jour. Lab. and Clin. Med.*, 1922, VII, 743.

tate indicates albumin. Care must be exercised not to add an excess of the ferrocyanid, as the albuminous coagulum may be redissolved. The test is positive with albumin in a dilution of 1:70,000 (Glaesgen), but, like the preceding tests, its delicacy depends much on the concentration of the urine in salts.

Sources of Error.—Albumoses and Bence-Jones protein are coagulated, but the coagulum disappears on heating—completely in the case of albumose, partially with Bence-Jones body.

Numerous other tests for the recognition of albumin in the urine have been devised. Some of them, as Spiegler's, are too delicate. The tests given above have been thoroughly tested, and are almost universally employed by clinicians. Thorough familiarity with them should be sufficient for all practical purposes.

Quantitative Determination of Albumin

Tsuchiya's Modification of the Esbach Method.[32]—Tsuchiya has devised a new reagent for precipitating the coagulable protein, to be used with the Esbach tube. The formula of Tsuchiya's reagent is:

Phosphotungstic acid	1.5 gm.
Hydrochloric acid, conc.	5.0 c.c.
Alcohol, 96 per cent, to 100.0	c.c.

Method.—If alkaline, the urine is acidified with a few drops of acetic acid to prevent bubbling, when the reagent is added. The Esbach tube is filled with urine to the mark U, and then the reagent is added to the mark R. The tube is corked and inverted twelve times to insure thorough and uniform mixing of the urine and reagent. (Do not shake, since bubbles clinging to the precipitate cause it to float.) The tube is placed in a vertical position for twenty-four hours at room temperature to allow the precipitate to settle, when the height of the precipitate is read on the scale marked on the tube. The figure obtained gives the quantity of albumin in grams *per liter.*

With large quantities of albumin the urine should be diluted with water, so that the reading will be below 4 gm. per liter, for Mattice has shown that above this the error increases greatly.

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[32] Mattice, A. F. "The quantitative estimation of albumin in the urine." *Arch., Int. Med.*, 1910, V, 313.

Tsuchiya's is a great improvement on the Esbach reagent, and should supplant it. With Esbach's reagent as the precipitant, the results are often not even approximately correct. Some of the advantages of Tsuchiya's reagent over that of Esbach are: (1) that the precipitate rarely floats, but (2) settles evenly in the bottom of the tube; (3) the readings are much less affected by slight variations in temperature; (4) the average error is very greatly reduced, amounting to less than 0.3 gm. per liter (controlled by the Kjeldahl and gravimetric methods), so that daily variations in albumin output can be followed with considerable accuracy, and (5) the reagent is clean, and does not stain hands or clothes (Mattice). Glucose in the urine does not interfere with the accuracy of the test.

Normal urines usually yield a slight precipitate when treated with Tsuchiya's reagent, but the bulk of it is so small that it is not measureable, and in no way interferes with the test.

Removal of Albumin from the Urine.—As albumin interferes with certain reactions, it is necessary at times to remove it before performing other tests. A convenient method is the heat and dilute acetic acid test. The coagulated protein is removed by filtration, and the filtrate tested by one of the other tests to determine whether it is albumin-free.

BENCE-JONES' BODY

Bence-Jones' proteinuria has been noted in:

1. Cases of *multiple myeloma.*
2. Some cases of *malignant disease* with metastatic involvement of the *bone marrow.*
3. Some cases of *leukemia.*
4. Severe *renal insufficiency* (uncommon).[33]

This protein is of rare occurrence. There is no simple, decisive, qualitative test by which it may be recognized. Its presence may be strongly suspected, though not absolutely proved, by the following reactions: If alkaline or neutral, acidify the urine with dilute acetic acid; filter the specimen, if necessary, to render it clear. (1) On heating the urine slowly in a test tube there appears a milky turbidity at about 52° C.; at 60° C. the precipitate is abundant and sticky. After the temperature rises above 70° C. the precipitate usually lessens

[33] Barker, C. E. "Bence-Jones proteinuria associated with severe renal insufficiency." *The Clinic Bulletin* (Mayo Clinic), 1922, III, No. 180.

materially, and may entirely disappear at the boiling point, though a slight cloud usually persists. As the urine cools, the precipitate reappears. (2) The addition of an excess of nitric acid to the cold urine causes a precipitate, which is partially or completely dissolved on boiling, but again separates as the temperature becomes lower. (3) Similar reactions may be obtained with many of the tests for albumin. That all of these reactions are influenced very greatly by the acidity and the salt content of the urine has been shown by Massini.[34] The further identification of Bence-Jones' protein is more or less complicated; the reader is referred to the larger works or to the literature. Very rarely, Bence-Jones' protein precipitates spontaneously in the urine as crystals,[35] having flat, scalelike or needle-shaped forms.[36]

ALBUMOSE

Albumosuria is of practically no clinical importance.

The secondary or deutero-albumoses, though of wide occurrence in the urine in disease, are ordinarily of little diagnostic importance. Their presence may be shown in the following manner (Simon): Strongly acidify a few c.c. of urine with acetic acid, and then add an equal volume of saturated solution of sodium chlorid. The presence of albumose is indicated by the occurrence of a precipitate, which disappears on boiling and reappears on cooling. Since albumin is usually present in the urine with albumose, the boiling urine should be filtered to remove the albuminous precipitate. A cloud, which develops in the filtrate on cooling, signifies albumose. To the hot filtrate an excess of sodium hydrate is added to render it strongly alkaline, then 1 per cent copper sulphate drop by drop, when a red color appears (the biuret test).

GLUCOSE

(*Dextrose, Grape Sugar*)

Glucose is present normally in the urine in traces, the quantity varying between 0.015 and 0.04 per cent in the twenty-four-hour specimen. The amount is so small that it is not detected with the usual clinical tests.

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[34] Massini, R. "Untersuchungen bei einem Falle von Bence-Jones' scher Krankheit." *Deutsch. Arch. f. klin. Med.*, 1911, CIV, 29.

[35] Walters, W. "Bence-Jones proteinuria." *Jour. A. M. A.*, 1921, LXXVI, 641.

[36] Rowntree, L. G. Personal communication.

Glycosuria is usually the result of a transient or constant *hyperglycemia,* and has been found:

1. In *diabetes mellitus.*
2. After consumption of large quantities of sugar—*alimentary glycosuria.*
3. After *emotional disturbances* at times. Thus, it has been observed in cases of mental depression; it has also been found in students (about 17.5 per cent) after hard examinations.
4. In well marked cases of *hyperthyroidism,* glycosuria is common.
5. In *hepatic cirrhosis,* at times.
6. In *pituitary disease* at times, especially in the earlier stages of acromegaly.
7. In *diseases of the central nervous system* at times. Glycosuria has been detected in cases of general paresis, brain tumor, cerebral hemorrhage, skull fracture, tabes, cerebrospinal meningitis.
8. In certain cases of *obesity* (pituitary ?).
9. In some patients with *gout.*
10. After certain *poisons,* such as morphin, cocaine.

Glycosuria occurs *without hyperglycemia:*

1. In *"renal" diabetes.*[37]
2. At times after administration of *diuretics,* such as diuretin, caffein, theocin.
3. After administration of *phloridzin.*

The urine to be tested should be clear. Simple filtration may be sufficient. If this fails the urine is shaken with powdered normal lead acetate, and then filtered.

Qualitative Tests

1. **Benedict's Test.**[38]—The reagent is prepared according to the following formula and keeps indefinitely.

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[37] Marsh, P. L. "Renal glycosuria." *Arch. Int. Med.,* 1921, XXVIII, 54 (literature).

[38] Benedict, S. R. "The detection and estimation of glucose in urine." *Jour. A. M. A.,* 1911, LVII, 1193.

Copper sulphate (pure crystallized)	17.3 gm.
Sodium or potassium citrate	173.0 gm.
Sodium carbonate (crystallized) [39]	200.0 gm.
Distilled water to make.....	1,000.0 c.c.

The citrate and the carbonate are dissolved together (with the aid of heat) in about 700 c.c. of water. The mixture is then poured (through a filter, if necessary) into a larger beaker. The copper sulphate (which should be dissolved separately in about 100 c.c. of water) is then poured slowly into the first solution with constant stirring. The mixture is then cooled and diluted to one liter.

Method.—About 5 c.c. of the reagent are placed in a test tube and *8 to 10 drops* (*not more*) of the urine to be examined are added. The mixture is then heated to vigorous boiling, kept at this temperature for one or two minutes, and allowed to cool spontaneously. In the presence of glucose, *the entire body of the solution will be filled with a precipitate,* which may be red, yellow or greenish in tinge. If the quantity of glucose be low (under 0.3 per cent), the precipitate forms only on cooling. If no sugar be present, the solution either remains perfectly clear, or shows a faint turbidity that is blue in color, and consists of precipitated urates.

This is the best of the reduction tests and is delicate, indicating the presence of 0.01 to 0.02 per cent glucose.

The chief points to be remembered in the use of the reagent are (1) the addition of a small quantity of urine (8 to 10 drops) to 5 c.c. of the reagent, since more delicate results are obtained by this procedure, (2) vigorous boiling of the solution after the addition of the urine, and then allowing the mixture to cool spontaneously, and (3) if sugar be present, the solution (either before or after cooling) *will be filled from top to bottom with a precipitate,* so that the mixture becomes opaque. Thus, bulk, and not the color, of the precipitate is made the basis of a positive reaction. Since no strongly dehydrating substance like caustic alkali is present, the precipitate is apt to be yellow, or even greenish yellow (the hydrated suboxid of copper), rather than the red suboxid.

Sources of Error.—Chloroform, chloral, formaldehyd, aldehyd, uric acid and creatinin, all of which reduce Fehling's solution, are without appreciable effect on Benedict's solution. *Homogentisic acid* (from the

[39] One half the amount (100 gm.) of the anhydrous salt may be used.

urine of alkaptonurics) or *greatly increased amounts of glycuronic acid,* both of which substances readily reduce Fehling's solution, will also reduce Benedict's reagent. When the presence of these bodies is suspected, the urine should be tested before and after fermentation with yeast. If the reducing action of the urine persists after twenty-four hours' fermentation, the reduction is not due to glucose, but may be due to lactose or to either of the two substances mentioned above. Interference from homogentisic acid is exceedingly rare, and occurs from glycuronic acid only after the ingestion of drugs which lead to increased elimination of this substance (see p. 52).

2. **Fehling's Test.**

Solution (1): [40]

Copper sulphate, cryst.	34.64	gm.
Distilled water to	500.0	c.c.

Solution (2):

Rochelle salt	173.0	gm.
Sodium hydrate	125.0	gm.
Distilled water to	500.0	c.c.

More than a trace of albumin should be removed from the urine before testing. Equal volumes of solutions (1) and (2) are mixed [41] in a test tube and boiled; the deep blue fluid should remain perfectly clear. Now (*a*) add the urine in small amount, never exceeding one-half the volume of the mixed solutions originally taken. A yellow or red precipitate appears at once. A second way (*b*) of performing the test is to layer the urine over the mixed, boiled solutions by allowing it to run down the side of the test tube. At the line of contact a yellow precipitate, which quickly turns red and diffuses downward, is formed in the presence of glucose. The precipitate appears within a few seconds. With small amounts of glucose, the diffusion downward is lost, but the red oxid soon collects at the bottom of the tube. With the second procedure (*b*) there is less likelihood of confusion in interpreting the test.

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[40] If solution (1) is to be used for *qualitative work only,* it is not necessary to weigh the copper exactly on an analytical balance. In preparing solution (2), dissolve the Rochelle salt in hot water, then cool to room temperature, add the sodium hydrate and make up to 500 c.c.

[41] A mixture of the two solutions is not permanent, and should, therefore, always be *freshly prepared* at the time of performing the test.

The test is said to reveal 0.1 per cent of glucose (Benedict).

Sources of Error.—A dirty, greenish-yellow precipitate does not mean sugar in the majority of instances. A precipitate which appears on standing means nothing.

When the reduction of the copper is atypical, the interpretation of the result is in doubt. The *combined glycuronates, uric acid, creatinin, creatin,* are all capable of reducing copper to a certain extent. They never cause more than a dirty yellow; the granular, red precipitate of cuprous oxid is missed, for ammonia, creatinin, etc., keep in solution the small amounts of cuprous oxid formed in sugar-free urines. With *less than* 0.1 *per cent of glucose,* a similar result may be obtained, for the sugar itself may hold in solution a small quantity of cuprous oxid; on cooling the red, granular precipitate may appear. The *alkaptone bodies* may also cause an atypical reduction. *Other hexoses* or *pentoses* may be responsible for the reaction. Before testing with any copper solution, *chloroform* must be removed from the urine by boiling, as it is a fairly strong reducing agent. Urine preserved with *formaldehyd* may likewise give a reduction.

3. **Almén-Nylander's Test.**—Reagent. Four grams of Rochelle salt are dissolved in 100 c.c. of warm 10 per cent sodium hydrate. The mixture is saturated with bismuth subnitrate (add about 2.0 gm. of the latter), filtered, and placed in a dark bottle. The reagent is permanent.

Albumin must be removed from the urine, since the sulphid of bismuth, which may result from its presence, is brown and interferes with the test.

To the urine in a test tube add one-tenth volume of the reagent, mix, and place the tube in a boiling water bath for *five minutes.*[42] More prolonged boiling should be avoided, otherwise sugar-free urine may reduce the bismuth. If dextrose is present the fluid darkens, and a black precipitate of metallic bismuth separates. When the solution turns dark only on cooling, the test is negative. In a sugar-free urine a white precipitate of phosphate is formed.

The test indicates 0.08 per cent of glucose, maltose, or lactose, and 0.07 per cent of levulose (Rehfuss and Hawk).

Sources of Error.—Nylander's solution is *not* reduced by uric acid, creatinin, the alkaptone bodies, pyrocatechin, and phosphates, and the

[42] Rehfuss, M. E., and Hawk, P. B. "A study of Nylander's reaction." *Jour. Biol. Chem.,* 1909-10, VII, 273.

test is, therefore, a good control of Fehling's test. *Pentoses* may cause a reduction; the same is true of *hexoses*. The test may be positive after eating *asparagus,* and also after the administration of *hexamethylenamin* (urotropin). An excess of *combined glycuronates* may cause a reduction. *Chloroform* should be removed from the urine by boiling. *Formaldehyd,* when added to the urine, reduces the bismuth.

Rehfuss and Hawk agree with Kistermann that any protein-free urine which gives a negative Nylander's test may safely be said to be sugar-free in a clinical sense. It is safer than Fehling's test, and should be used more extensively than it is.

4. **The Fermentation Test.**—When positive, this test proves that the reducing body is a fermentable sugar. In the vast majority of instances the sugar is glucose.

A piece of fresh compressed yeast about the size of a hazel nut is rubbed in a mortar with about 50 c.c. of urine, which is then filled into a fermentation tube, so that the air is completely displaced. As controls, use (*a*) normal urine and yeast, and (*b*) normal urine and yeast plus glucose, to prove the activity of the yeast. The three tubes are set aside in a warm place (temperature 20° to 37° C.) for several hours. If the yeast is active and glucose present, alcohol and carbon dioxid gas will be evolved, the bubbles collecting at the top of the tube. No gas, or only a minute bubble, should be evolved in the control tube (*a*), whereas the glucose added to control tube (*b*) should be fermented. To lessen the danger of bacterial decomposition, the urine may be boiled before testing. The test will indicate 0.05 to 0.1 per cent of glucose. As a further check, the reduction tests may be repeated with the filtered urine after fermentation is completed.

A positive test indicates the presence of a fermentable sugar.

Sources of Error.—Levulose and *maltose,* if present, may be fermented with the evolution of gas. Before adding the yeast *chloroform* must be removed from the urine by boiling. *Thymol* and *formaldehyd,* when used as preservatives, may inhibit the growth of the yeast. It is said that *hexamethylenamin* in sufficient doses also prevents the fermentation.

5. **Cippolina's Modification of the Phenylhydrazin Test.**[43]—Albumin, when present, should be removed before performing the test.

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[43] Cippolina, A. "Ueber den Nachweis von Zucker im Harn." *Deutsche med. Wchnschr.,* 1901, XXVII, 334.

To 4 c.c. of urine in a test tube are added 5 drops of pure phenylhydrazin (the base) and 0.5 c.c. of glacial acetic acid (or 1.0 c.c. of 50 per cent acetic acid); the mixture is boiled gently over a low flame for one minute. Now add 4 to 5 drops of sodium hydrate (sp. gr. 1.160); the mixture must still remain acid. The whole is heated a few seconds longer, and set aside to cool. Immediately or within about twenty minutes, especially with a urine of low specific gravity, the characteristic sheaf-like yellow needles of phenylglucosazone (Fig. 5) appear. Since their size is subject to considerable variation, high magnification is necessary at times to see them.

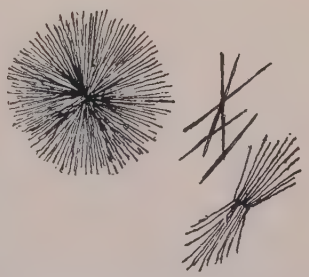

Fig. 5.—Crystals of Phenylglucosazone. Phenylhydrazine test.

The test is very delicate, indicating 0.05 per cent of glucose. However, the sensitiveness of the test depends very largely upon the specific gravity of the urine. Concentrated urines may react negatively in the presence of less than 0.2 per cent of glucose. The reason for this is that phenylglucosazone crystals are held in solution in the presence of much urea, ammonium salts, and other nitrogenous bodies. But with more than 0.2 per cent of glucose typical crystals form within a few minutes, regardless of the specific gravity of the urine.

In place of the characteristic needles, yellow balls, which change to thorn-apple forms or rosettes, may be obtained. The latter are seen only in urine containing a pathological quantity of sugar, never in a normal urine (Cippolina). Characteristic needles arranged in sheaves may be obtained by recrystallization from hot 60 per cent alcohol. When the crystals are atypical the specimen should be set aside and reëxamined at the end of one hour.

To determine definitely that the crystals are derived from glucose and not from another sugar, it is necessary to filter them off and purify them by repeated recrystallization from hot 60 per cent alcohol. (The melting point of the purified crystals may be determined;[44] that of phenylglucosazone is 204 to 205° C. The melting point of levulosazone is the same, while maltosazone crystals melt at about 207° C. The value of melting point determinations for the identification of one of the three

[44] For a description of methods, with critical discussion, see Menge, G. A. "A study of melting-point determinations." *Bull. No. 70,* Hyg. Lab., U. S. Pub. Health & Mar. Hosp. Serv., Wash., 1910.

sugars mentioned is, therefore, not great, though very helpful in differentiating the osazone of pentose, melting point 168° C., somewhat less so with lactose, 200° C.)

The dry, purified crystals may be identified by dissolving 0.2 gm. of them in 4 c.c. of pure pyridin, to which 6 c.c. of absolute alcohol are subsequently added, and the whole well mixed. The 100 mm. tube of the polariscope is then filled with this mixture. Phenylglucosazone gives a *levorotation* of—1° 30′. This procedure is seldom, if ever, necessary in clinical work.

Quantitative Estimation of Glucose

1. **Benedict's Method.**[45]—This is the best quantitative method for the clinician. Benedict's directions for the preparation of the solution and for the titration follow:

Crystallized copper sulphate	18.0 gm.
Anhydrous sodium carbonate[46]	100.0 gm.
Sodium citrate	200.0 gm.
Potassium sulphocyanate	125.0 gm.
Five per cent potassium ferrocyanid solution	5.0 c.c.
Distilled water to	1,000.0 c.c.

With the aid of heat dissolve the citrate, carbonate, and sulphocyanate in enough water to make about 800 c.c. of the mixture, and filter. Dissolve the copper sulphate separately in about 100 c.c. of water, and pour the solution slowly into the other liquid, with constant stirring. Add the ferrocyanid solution cool, and dilute to exactly one liter. Of the various constituents, *only the copper sulphate need be weighed with exactness.* Twenty-five c.c. of the reagent are reduced by 0.050 gm. of *glucose,* or by 0.053 gm. of *levulose.*

Method.—With a pipette measure 25 c.c. of the reagent into a porcelain evaporating dish (25 to 30 cm. in diameter) and add 5 to 10 gm. of anhydrous sodium carbonate (or twice the weight of the crystallized salt), and a *very small quantity* of powdered pumice stone. Heat the mixture to vigorous boiling over a free flame till the carbonate is dis-

[45] Benedict, S. R. "A method for the estimation of reducing sugars." *Jour. Biol. Chem.,* 1911, IX, 57.

[46] 200.0 gm. of the crystallized salt may be used.

solved, and from a burette run in the twenty-four-hour specimen of urine (diluted accurately 1:10, unless the sugar content is known to be very slight) quite rapidly, until a heavy white precipitate (cuprous sulphocyanid) is produced, and the blue color of the solution begins to diminish perceptibly. From this point the urine is run in more and more slowly, with constant vigorous boiling, until the disappearance of the last trace of blue color, which marks the end-point. An interval of 30 seconds' vigorous boiling should be allowed between each addition of urine.

Example.—Let us assume that the patient has voided 3,600 c.c. of urine in the twenty-four hours and, further, that 15 c.c. of urine (diluted 1:10) are required to completely reduce 25 c.c. of the quantitative Benedict's solution, equivalent to 0.05 gm. glucose. Then, $\frac{3,600}{15} \times 0.05 \times 10$ (= dilution) = 120 gm. glucose, the quantity passed by the patient in 24 hours.

(The sugar excretion should always be expressed in *grams per diem,* since percentage means nothing unless one knows the total quantity of urine voided.)

The following explanatory points may be added regarding the solution: When ready mixed, the solution appears to keep indefinitely without any special precaution, such as exclusion of light, etc. The trace of ferrocyanid is added to prevent precipitation of red cuprous oxid, which may be caused by certain impurities. *Chloroform has such a marked tendency in this respect that it must not be present during the titration.* The additional alkali is added prior to the titration in order to provide sufficient alkalinity to insure a sharp end-point. Should the mixture become too concentrated during the titration process, distilled water may be added to replace the volume lost by evaporation.

2. **Polariscopic Determination.**—The polariscope is an expensive instrument, and for this reason it is not as generally employed for sugar determinations as the rapidity and ease of its use would seem to warrant. For clinical use the instrument is supplied with two specially made tubes, 94.7 mm. and 189.4 mm. long, which permit a direct percentage reading of glucose; the short tube is used with dark, highly colored urines, the readings obtained being divided by two. The tubes must be perfectly clean and dry before using; hot water or fluid should not come in contact with them, since the expansion of the glass against the outer brass tubing may crack the former.

The twenty-four-hour specimen, *acid* in reaction, is filtered and decolorized, if necessary. This is best accomplished by the addition of about 2 gm. of finely powdered normal lead acetate to the urine, which is then shaken vigorously and filtered. The first cloudy portions of the filtrate are returned to the filter, until the filtrate, which is almost colorless, is perfectly clear. Practically no sugar is held back by the normal lead acetate.[47] The clear urine is now filled into the polariscope tube (189.4 mm. in length) until the fluid is convex above the end of the tube. The glass disc is then placed over the end of the tube and secured in place by screwing down the metal cap. Air bubbles must be avoided, since their presence makes a satisfactory reading impossible. The tube is now placed in the polariscope, which must be in a dark room. For illumination a sodium flame is used. After focusing, readings are made, first without the urine, to determine whether the zero point is accurate, next, after refocusing, with the tube of urine; starting at zero, the handle is rotated until the entire field is equally illuminated. At least six readings should be made. The percentage is read directly from the scale, tenths being obtained on the vernier. (In case the instrument is supplied only with the standard tubes of 100 and 200 mm. length, the percentage of glucose may be calculated from the polariscopic readings by dividing the results by 0.527.)

The method gives fairly satisfactory results. When no disturbing bodies are present in the urine, the error is about 0.1 per cent of glucose.

Sources of Error.—(1) *Albumin,* when present, must be removed before making polariscopic determination of glucose, otherwise the albumin, which is levorotatory, will counterbalance the dextrorotatory glucose, in part at least. (2) *Alkalinity* of the urine precludes its use with the polariscope, since it has been shown that in alkaline media dextrose may be converted into levulose.[48] The addition of a preservative to the specimen usually suffices to prevent an acid urine becoming alkaline. (3) *β-oxybutyric acid* is levorotatory, and its presence, therefore, interferes with the accurate estimation of glucose. (4) The *combined glycuronates* are levorotatory, though they are generally present in such small quantity as to produce only slight rotation of the polarized light. (5) *Levulose,* when present in the urine with glucose, is antag-

[47] Neuberg, C., II. "Ueber Klärung und Entfärbung." *Biochem. Ztschr.,* 1910, XXIV, 423.

[48] Koenigsfeld, H. "Zur Klinik und Pathogenese der Lävulosurie beim Diabetes mellitus." *Ztschr. f. klin. Med.,* 1910, LXIX, 291.

onistic, and lowers the reading for glucose. (6) *Maltose* is occasionally present in the urine with glucose. Since it is more powerfully dextrorotatory than glucose, the reading may give a value which is too high.

From the foregoing it is apparent that the error arising from β-oxybutyric acid may be estimated approximately by making polariscopic examination of the specimen before and after fermentation with yeast. With the combined presence of glucose and levulose, the relative proportions of each may be determined with a fair degree of accuracy by comparison of the value obtained by titration with copper solution and the polariscopic value.

3. **Robert's Specific Gravity Method.**[49]—This method depends upon a lowering of the specific gravity of the urine as a result of fermentation of the sugar. By obtaining the specific gravity of the fermented and the unfermented urine, the quantity of sugar may be calculated. About 2.0 gm. of compressed yeast are rubbed in a mortar with 50 c.c. of the twenty-four-hour specimen of urine, acidified with acetic acid if necessary. The specific gravity of the suspension is taken at once, the temperature of the mixture being noted. The mixture is set aside in a warm temperature (25 to 37° C.) in a receptacle plugged with cotton, or, better, in a large fermentation tube. When fermentation is complete the yeast settles to the bottom of the flask; it is well, nevertheless, to test the fluid to determine the complete disappearance of the sugar. The mixture is well stirred and a small portion removed. It is filtered and tested with Fehling's solution. If glucose still remains, the fermentation is allowed to continue, until there is no longer a reduction of the copper. The mixture is again stirred thoroughly to restore the suspension, and the specific gravity is determined the second time. It is important that the temperature of the suspension at the times of determining specific gravity does not differ by more than 1° C. The usual urinometers are too inaccurate for the determination of the specific gravity, which should be carried to the fourth decimal place. Lohnstein's instrument (Fig. 6) is convenient and satisfactory for the purpose. It is an aërometer, in whose stem the pan, *A*, is mounted. It is floated in the urine and weights are placed on the pan until the shelf, *C*, is exactly on a level with the surface of the fluid. The sum of the weights on the pan is the specific gravity of the fluid. If the pan is loaded too heavily, so that the surface, *C*, sinks into the fluid, it must be removed and dried.

[49] Lohnstein, Th. "Ueber die densimetrische Bestimmung des Traubenzuckers im Harne." *Arch. f. d. ges. Physiol.*, 1895-6, LXII, 82.

The quantity of sugar is calculated by multiplying the difference in the specific gravities by the factor 234. The result is glucose in grams per

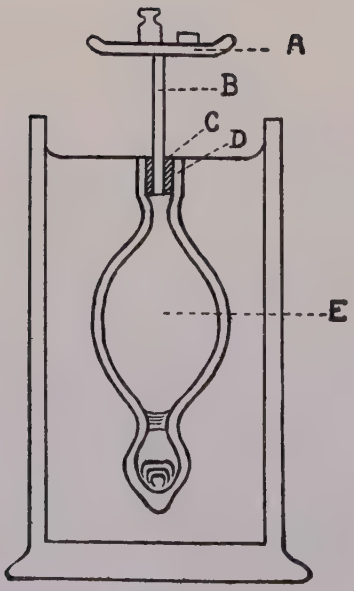

Fig. 6.

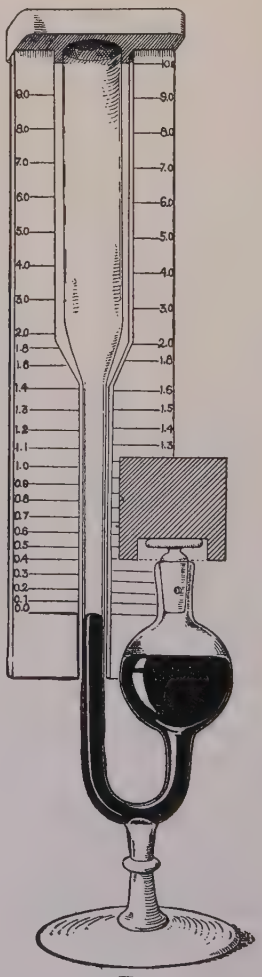

Fig. 7.

Fig. 6.—Lohnstein's Aërometer. *A*, pan for weights; *C*, shelf which should be level with the surface of the fluid; *E*, air chamber (after Lohnstein).

Fig. 7.—Lohnstein's Fermentation Saccharimeter for Undiluted Urine (after Wood).

cent. When unfiltered urine is used (that is, for the second determination, after fermentation is completed), the error does not exceed 5 per

cent (Lohnstein). The method permits the determination of glucose in strengths of 0.1 per cent or more.

4. **Measurement of the carbon dioxid gas formed during fermentation** has been used to determine the dextrose content of urine. One of the most widely known forms of apparatus for this purpose is Einhorn's. More accurate results have been obtained with Lohnstein's apparatus (Fig. 7). This consists of a J-shaped tube mounted on a stand. In the short arm of the tube a bulb is blown, the outlet of which may be closed by a glass stopper. A hole in the neck may be brought opposite a similar opening in the hollow stopper. The long arm is provided with a scale. A quantity of mercury and a pipette for measuring the urine are supplied with the apparatus.

Method.[50]—The mercury is poured into the apparatus. The bulb is partly filled, and mercury extends a short distance up the long arm of the tube. Then, with the pipette, 0.5 c.c. of urine and 0.1 to 0.2 c.c. of a yeast suspension[51] (1 part of compressed yeast to 2 to 3 volumes of water) are placed in the bulb on the surface of the mercury. The glass stopper (greased with vaselin 20 per cent, in yellow wax) is placed in the neck of the bulb in such a way that the openings are opposite one another; this is to avoid a positive pressure on inserting the stopper. The scale is now placed on the long arm of the tube; the zero line should correspond with the level of the mercury in the long arm. The stopper is turned, to close the bulb, and a weight (provided with the apparatus) is placed over it to prevent it from being blown out, as the carbon dioxid forms. The apparatus is placed in an incubator at a temperature of 32 to 38° C. for 4 to 5 hours, or at room temperature for 24 hours. The carbon dioxid formed in the bulb from fermentation of the sugar forces the mercury into the long arm of the tube. The percentage of glucose is read directly from the scale, which is provided with two columns of figures, one for the average room temperature, the other for body heat.

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[50] Lohnstein, T. "Ueber Gärungs-Saccharometer nebst Beschreibung eines neuen Gärungs-Saccharometers für unverdünnte Urine." *München. med. Wchnschr.*, 1899, XLVI, 1671.

[51] The quantity of yeast suspension employed depends upon the concentration of glucose. With very low percentage of sugar the yeast may be rubbed up with 10 to 15 volumes of water.

LEVULOSE

Levulose, when present in the urine, is usually associated with dextrose. Occasionally it is the only sugar in the urine, a few cases of levulosuria having been reported.[52]

Levulosuria [53] has been met with:

1. In *diabetes mellitus,* levulose being present only in traces, with glucose.
2. In *"urinogenous" levulosuria;* glucose in an alkaline urine may be inverted to levulose.
3. In *spontaneous alimentary levulosuria,* in which levulose is the only sugar present in the urine (up to 1.8 per cent).
4. In *alimentary levulosuria,* after ingestion of large quantities of levulose, especially in diseases of the liver.

Levulose responds to most of the tests for glucose. It is fermentable, reduces copper and bismuth, gives the phenylhydrazin test; there are, however, certain dissimilarities by which the two sugars may be separated.

1. **Seliwanoff's Test, as Modified by Borchardt.**[54]—Five to 10 c.c. of urine and an equal volume of 25 per cent hydrochloric acid (i. e., 2 parts of concentrated hydrochloric acid and one part of water) are mixed, and a few grains of resorcin added. The mixture is boiled gently for a few seconds. A red color appears, usually followed by a brownish precipitate, if levulose is present. The fluid is now cooled, poured into an evaporating dish or beaker, and treated with sodium carbonate in substance until the reaction of the mixture becomes alkaline. It is then returned to a test tube, and shaken with acetic ether (ethyl acetate). In the presence of levulose the acetic ether is colored yellow. The test indicates 0.05 per cent of levulose.

Sources of Error.—(1) The simultaneous presence of nitrites and indican in considerable quantity may yield a positive reaction. The nitrites may be destroyed by acidifying the urine with acetic acid and boiling for one minute. (2) Large quantities of indican alone may interfere with the reaction by imparting a blue color to the acetic

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[52] Strouse, S., and Friedman, J. C. "Lævulosuria." *Arch. Int. Med.,* 1912, IX, 99.

[53] Koenigsfeld, H. *Loc. cit.,* p. 42.

[54] Borchardt, L. "Ueber die diabetische Lävulosurie und den qualitativen Nachweis der Lävulose im Harn." *Ztschr. f. physiol. Chem.,* 1908, LV, 241.

ether, making it impossible to recognize the yellow color from levulose. In such case the indican is removed by treating the urine with an equal volume of Obermayer's reagent and extracting several times with chloroform, until the latter is no longer colored blue. The fluid is then poured into a fresh test tube, the chloroform being discarded, and is diluted with one-third volume of water, in order to reduce the strength of the hydrochloric acid to 12 to 13 per cent. A knife point of resorcin is now added, and the Seliwanoff test carried out. (3) Urorosein, when abundant in the urine, may impart a reddish-violet color to the acetic ether. To remove the pigment before applying Seliwanoff's test, take equal quantities of urine and 25 per cent hydrochloric acid, and extract the mixture two to three times in a separating funnel with amyl alcohol. Discard the amyl alcohol, which contains the urorosein, add resorcin, and proceed with the test in the usual way. (4) It has been found that patients taking santonin or rhubarb may give a positive Seliwanoff reaction. Discontinuance of the drug causes the reaction to disappear.

In performing the test prolonged boiling should be avoided. Borchardt finds no interference with the reaction from the presence of glucose, lactose, maltose, arabinose, or glycuronic acid compounds. Saccharose may yield a positive reaction, since boiling it with acid liberates levulose.

2. **The Phenylhydrazin Test.**—This test (see p. 38) gives crystals of phenyllevulosazone. They are indistinguishable microscopically from phenylglucosazone; the melting point of each is the same. The crystals can be positively identified by determining their rotation of polarized light. They are purified by repeated crystallization from hot 60 per cent alcohol. Then 0.2 gm. of the pure crystals are dissolved in 4 c.c. of pyridin, and 6 c.c. of absolute alcohol are added. The mixture is poured into the 100-mm. tube of the polariscope and examined. Levulosazone gives a *dextrorotation* of 1° 20′.

Levulosuria combined with glycosuria should be suspected when the quantity of glucose found on polariscopic examination falls short of that shown by titration. A positive Seliwanoff reaction and the lack of a levorotatory body after fermentation practically confirm it.

Pure levulosuria offers no difficulties in recognition, if access to a polariscope may be had. The levorotation of the urine, together with positive Seliwanoff and reduction tests and the presence of a fermentable substance, makes the identification sufficiently complete, if all tests are negative after fermentation. The phenylhydrazin test, as described

above, removes all doubt, if positive. Levulosuria is usually unsuspected, unless the urine be examined with a polariscope.

Alimentary levulosuria has been used in hepatic diagnosis. In the absence of derangement of hepatic function an individual can take 100 gm. of levulose on a fasting stomach without the subsequent appearance of levulose in the urine, as a general rule. On the other hand, the majority of patients with liver disease exhibit levulosuria under such conditions.[55]

MALTOSE

Maltose is occasionally present in the urine, usually in association with glucose. It reduces copper and bismuth solutions, and is fermentable. Maltose has about two and one-half times the dextrorotatory power of glucose, whereas its reducing power is only about two-thirds that of glucose. Therefore, its presence may be suspected when polariscopic values exceed the results found with the reduction methods.

LACTOSE

Lactose may appear in the urine of women physiologically in connection with stasis of milk in the breasts.

Lactosuria may be encountered:

1. Before or after *labor.*
2. Following *premature weaning,* from stagnation of milk in the breasts.
3. After *consumption of* excessive quantities of *lactose* (over 120 gm.) or very large quantities of *milk.*

At times in pregnant women glucose and lactose co-exist in the urine. In any case, it is important to determine the nature of a reducing substance in the urine in pregnancy, since lactose is without pathological significance and the reverse is true of glucose. Mathews[56] has devised a method, which is rapid and simple. The essential basis of the method is to ferment the urine with a *large amount* of yeast. The glucose is quickly fermented away and its reducing action is destroyed, while the lactose remains unaffected in the filtrate. Yeast ferments

[55] For a discussion of this test, see Churchman, J. W. "The Strauss test for hepatic insufficiency." *Bull. Johns Hopkins Hosp.,* 1912, XXIII, 10.

[56] Mathews, A. P. "An easy method for the distinction and estimation of lactose and glucose in urine." *Jour. A. M. A.,* 1920, LXXV, 1568.

only glucose and levulose, of those sugars that may be present in the urine.

Mathews' Method.—"The urine having been tested in the usual way by Fehling's solution or Benedict's solution and found to contain a reducing substance, 10 c.c. of urine are measured into a test tube and one-quarter of a cake of compressed yeast (Fleischmann's) is added to the urine, the test tube closed with a cork or the thumb, and thoroughly shaken until the yeast is uniformly distributed and there are no longer any separate lumps. The uncorked tube is then placed in an inclined position at an angle of about 45 degrees in a beaker or pan of water heated to 40 to 43° C., the tube being immersed in the water above the level of the urine. There will be a little air in fine bubbles which became mixed during the shaking. This will come to the surface and ascend the slanting side of the tube, but this will cease in a minute if there is no fermentation. If glucose or levulose is present, there begins within a couple of minutes a fermentation, the bubbles of gas rising to the slanting upper side of the test tube. With less than 1 per cent glucose, the fermentation is scarcely visible, but with more than this a steady stream of bubbles is given off. With 3 per cent or more, the fermentation is stormy. By this evolution of gas one can say within five or ten minutes that there is more than 1 per cent of glucose present. If there is no visible evolution of gas, it is certain that the amount of dextrose is less than 1 per cent, if it is present at all.

"In order to make the method still more precise, the urine is permitted to stand in contact with the yeast for fifty minutes at the temperature of from 40 to 43° C., then removed from the water bath, the yeast filtered off, and the filtered urine tested for reducing power with Benedict's or Fehling's solution. If it still reduces, it shows that there is some reducing substance there other than glucose. Glucose, even up to 6 per cent in the urine, is completely destroyed by this amount of yeast at this temperature in fifty minutes (five per cent or less is destroyed in thirty minutes).

Quantitative Determination.—"To make a quantitative determination of the amount of glucose and lactose present, the total amount of reducing action of the urine, considered as glucose, is first determined by titration by the Benedict method. Then 20 c.c. of urine are mixed with half a cake of yeast, as described above, and placed in a beaker of water at 42° C. for fifty minutes, the tube being inverted twice during this period to mix the yeast. At the end of this time, all glucose

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will have been destroyed by the yeast. Filtration is performed through a dry, folded filter. The filtrate will probably be opalescent, but this does not interfere.

"If the filtrate still gives a reduction, the lactose in 10 c.c. of the filtrate is determined by titration with Benedict's method.

"The difference between the figures for the titration before and after fermentation will give the amount of glucose present.

"To identify the lactose positively, it will be necessary to make the osazone [*see* phenylhydrazin test, p. 38] in a small portion of the urine after it has been treated with yeast and the glucose destroyed in the manner indicated."

Cupric salts are reduced more slowly than by glucose. Ammoniacal silver nitrate is reduced by lactose in the cold. Lactose is not fermentable by yeast. If equivocal results are obtained with the usual compressed yeast, the fermentation may have been due to contaminating bacteria. With a pure culture of Saccharomyces apiculatus, lactose is not fermented.

SACCHAROSE

Saccharose is seldom encountered in the urine. It is dextrorotatory. After inversion (heat 75 c.c. of the urine with 5 c.c. conc. HCl for five minutes at a temperature between 68 and 70° C.), the fluid becomes levorotatory, since the levulose which is formed more than neutralizes the glucose. Reduction tests become positive after inversion of the sugar.

PENTOSE

Pentose is rarely found in the urine. Pentoses are sugars with five carbon atoms.

Pentosuria may be observed, according to Janeway:

1. After *ingestion of large quantities of vegetables and fruits* containing pentosanes, as, for example, apple juice. Such pentoses are optically active.
2. In rare cases of *diabetes mellitus.*
3. In *chronic pentosuria,* occurring without reference to the pentoses of the food, and persisting unchanged for years. The pentose excreted in these cases is the optically inactive r-arabinose. Its concentration in the urine is generally between 0.2 and 0.6 per cent.

The only pentose of importance in the urine—r-arabinose—is, unlike other sugars, optically inactive. It reduces copper and bismuth solutions slowly and incompletely; with Nylander's solution a grayish precipitate may be obtained. Pentose does not ferment with yeast. Pentose should be suspected when the reduction tests are atypical, when they persist after attempts at fermentation, when the urine is inactive on polariscopic examination. The following tests may also be employed:

1. **The Phloroglucin Test.**—To about 5 c.c. of urine in a test tube add an equal volume of concentrated hydrochloric acid and a liberal knife-point (ca. 30 mg.) of phloroglucin. The mixture is heated, preferably on a water bath. A red color appears, and, soon afterward, a dark precipitate forms. The contents of the test tube are cooled, and are then extracted with amyl alcohol. Spectroscopic examination of the amyl alcohol extract reveals a band midway between D and E, a little to the right of the sodium line.

Sources of Error.—Glycuronic acid compounds yield a positive phloroglucin test, including the absorption band, thus lessening greatly the value of the test. Lactose and galactose give the same color reaction as pentose, but the characteristic absorption spectrum is lacking.

2. **The Orcin Test.**—Equal parts of the urine and concentrated hydrochloric acid (sp. gr. 1.19) and a small knife-point of orcin are boiled gently. If pentose is present a dark greenish color soon develops, and, finally, a turbidity, due to a dark blue or green precipitate. The contents of the test tube are cooled, until they are *lukewarm*, and are then extracted with amyl alcohol. The latter exhibits a dark, olive-green color, the depth of which depends largely upon the concentration of pentose in the urine. If the fluid is cold instead of lukewarm when extracted, the amyl alcohol is reddish and the absorption bands are not so plainly visible (Salkowski). Spectroscopic examination reveals a band at D, the sodium line.

Sources of Error.—The orcin test is also given by the paired glycuronic acid compounds. However, the latter react with orcin less readily than with phloroglucin, so that of the two the orcin test is to be preferred. It has been shown [57] that filter paper may contain pentose-like substances, which are soluble in hydrochloric acid. The urine should, therefore, not be passed through filter paper. Glass wool or asbestos should be employed in its stead.

[57] Umber, F. "Notiz über Pentosenreactionen in filtrirten Flüssigkeiten." *Berlin. klin. Wchnschr.*, 1901, XXXVIII, 87.

3. **Bial's Modification of the Orcin Test.**[58]

Reagent:

Orcin	1.0	gm.
30 per cent hydrochloric acid	500.0	c.c.
10 per cent ferric chlorid	25	drops

Keep the reagent in a dark bottle.

Method.—Heat about 4 c.c. of the reagent to boiling and then add a few drops of the urine to be tested. With pentose a green color develops immediately or in a few seconds. The quantity of urine employed should not exceed 1 c.c. Performed in this way, the test reacts only with pentose, not with paired glycuronic acid compounds (Bial).

The green fluid is extracted with amyl alcohol and examined spectroscopically, as in the orcin test.

The specificity of the test has been questioned by a number of observers.

GLYCURONIC ACID

Glycuronic acid (glucuronic acid) does not appear as such in the urine, but becomes paired or conjugated in the body with various substances, such as indoxyl, skatoxyl, phenol, in which form it is excreted in the urine. In small quantity it is normally met with. Glycuronic acid also combines with numerous drugs. Urochloralic acid, the chloral hydrate compound, is an example.

Glycuronic acid may be *present in the urine in increased quantities* after the administration of chloral, camphor, menthol, turpentine, sandalwood oil, carbolic acid, acetanilid, phenacetin, salicylic acid, etc. Increased quantities, through their power of reducing copper solutions, may be mistaken for glucose. The chloral compound, urochloralic acid, is the one most frequently leading to confusion.

With copper solutions the glycuronic acid compounds may give a slow, atypical reduction, often a greenish-yellow precipitate—a reaction quite like that produced by pentose or by very weak solutions of dextrose. The Nylander test may be positive. The phloroglucin and orcin tests are given by the combined glycuronates. The glycuronates do not give the phenylhydrazin test of Cippolina, though the test may become posi-

[58] Bial, M. "Ueber die Diagnose der Pentosurie mit dem von mir angegebenen Reagens." *Deutsche med. Wchnschr.*, 1903, XXIX, 477.

tive if the urine be boiled previously with 1 per cent sulphuric acid to liberate glycuronic acid; the crystals obtained melt at 114° to 115° C. The combined glycuronates do not ferment with yeast. It happens, therefore, that, when the urine contains abnormal quantities of both glucose and glycuronates, fermentation does not cause a complete loss of reducing power. The glycuronic acid compounds are levorotatory in acid urine (inactive if the reaction is alkaline), whereas glycuronic acid itself is dextrorotatory. Therefore, boiling one to five minutes with 1 per cent sulphuric acid changes a levorotation to dextrorotation, or, if glucose be present, it may increase the dextrorotation, provided some of the sugar is not destroyed. Pentose (r-arabinose) is optically inactive. β-oxybutyric acid is levorotatory. To distinguish between the levorotation produced by this acid and that due to the combined glycuronates, the urine is precipitated with subacetate of lead and filtered. The glycuronates are precipitated, while β-oxybutyric acid appears in the filtrate, where its presence may be indicated by polariscopic examination. Or, the β-oxybutyric acid may be extracted by shaking the urine with ether three or four times, the glycuronates remaining in the urine.

Normal urine may contain enough levorotatory substances to produce 0.1 degree of levorotation; when the glycuronates are increased, the levorotation is 0.2 degree or more.

B. Tollens' Test.[59]—To 5 c.c. of urine in a test tube add a bit of naphthoresorcin about the size of a millet seed and then 5 c.c. of concentrated hydrochloric acid (sp. gr. 1.19). Boil the mixture gently about one minute, and set the tube aside for about four minutes. Now cool the contents of the tube under running water. Extract with an equal volume of ether. (The separation of the ether may be hastened by the addition of a few drops of alcohol.) If glycuronates are present in the urine in excess, the ether extract is dark blue to violet, while with smaller amounts a faint bluish or reddish violet color is obtained. Examined spectroscopically, the ether extract shows a single dark band near the sodium line. (The examination should be made at once,[60] as the substance giving rise to the band is not stable.) In place of

[59] Tollens, C. "Ueber den Glykuronsäuren Nachweis durch die B. Tollensche Reaktion mit Naphthoresorcin und Salzsäure." *München med. Wchnschr.*, 1909, LVI, 652.

[60] Brooks, B. Personal communication.

naphthoresorcin in substance, 0.5 c.c. of a 1 per cent alcoholic solution of naphthoresorcin may be substituted. The test is sufficiently delicate to detect the small quantities of glycuronates present in normal urine.

The dark pigments formed in this reaction by pentoses and other sugars are insoluble in ether.

ALKAPTONURIA

Alkaptonuria [61] is a very rare condition, a disturbance in metabolism. Of the alkapton bodies, two, homogentisic acid and uroleucinic acid, have been isolated. When present in the urine they may give to it the following characteristics: The fresh urine is markedly acid. It is normal in color when voided, but on standing, oxidation quickly changes the color to a reddish-brown and, finally, to a black. The color changes occur more rapidly when the reaction of the urine is alkaline. The urine reduces copper and silver (the latter in the cold) but not bismuth. The urine does not give the phenylhydrazin test, does not rotate the plane of polarized light, and is not fermentable.

ACETONE

Acetone, a ketone, occurs in normal urine in amounts as high as 10 mg. in twenty-four hours. It is a colorless, odorless liquid, very volatile, of low specific gravity.

Acetonuria may be found:

1. In many *fevers.*
2. In *starvation.*
3. In some cases of *cancer.*
4. In certain *gastro-intestinal diseases,* especially when vomiting is excessive.
5. After *ether and chloroform anesthesia* at times.
6. After *extirpation of the pancreas.*
7. During the first two days of the *puerperium* at times; occasionally during the latter part of *pregnancy.*
8. In *diabetes mellitus.*

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[61] For full discussion and a review of the recent literature, see Gibson, R. B. and Howard, C. P. "A case of alkaptonuria with a study of its metabolism." *Arch. Int. Med.,* 1921, XXVII, 632.

In testing the urine for acetone, it is usually necessary to distil the specimen. Occasionally, when very large quantities of acetone are present, positive reactions for acetone may be obtained by testing the urine directly. But in no case do such tests, when negative, exclude an acetonuria. When tests of the urine are negative, it becomes necessary to distil a portion of it and *to apply the tests for acetone to the distillate.*

Between 200 and 300 c.c. of urine are acidified with 1 to 2 c.c. of concentrated hydrochloric acid[62] and distilled. The greater part of the acetone is contained in the first 20 or 30 c.c. of the distillate, which is used for the tests to be described. If distillation of the urine is impossible, about 50 c.c. of urine are extracted with 20 c.c. of ether in a separating funnel. The urine is then allowed to escape, and to the ether about 10 c.c. of water are added. The fluids are well shaken. A large part of the acetone is in the water, which is then used for the qualitative tests.[63]

Qualitative Tests

1. **Gunning's Test.**—Five c.c. of the distillate are rendered alkaline with 5 to 10 drops of ammonium hydrate, and then Lugol's solution (potassium iodid, 6 gm., iodin, 4 gm., distilled water to 100 c.c.) or tincture of iodin is added until the deep black precipitate which forms no longer dissolves at once. This gradually disappears and is replaced by a yellow precipitate of iodoform crystals (Fig. 8), recognized by their characteristic odor and morphology. The crystals are often so small that the high-power dry objectives of the microscope are required. They are hexagonal plates, often clustered in the form of six-pointed stars. When atypical, the crystals should be recrystallized from alcohol-free ether. They are colored yellow. When the test is applied directly to the urine the phosphates are precipitated by the ammonia, usually in the form of crystals resembling fern leaves. With very small quantities of acetone it may

Fig. 8.—Iodoform Crystals. Gunning's test for acetone.

[62] Phosphoric acid may be used. The acid is added only to prevent the distillation of ammonia and excessive foaming of the urine.

[63] Bohrisch, P. *Pharm. Zentralhalle,* 1907, XLVIII, 5; 184; 206; 220; 245. Cited by F. N. Schulz in Neubauer-Huppert's *Analyse des Harns,* 11th Ed., p. 252. Wiesbaden, 1910.

be necessary to wait twenty-four hours for the crystals of iodoform to form.

This test is the best qualitative test, since a positive reaction is obtained only with acetone. It is slightly less sensitive than Lieben's test. According to Bohrisch, the test should be applied only to the distillate, not to the urine directly.

2. **Lieben's Test.**—A few drops of 10 per cent sodium or potassium hydrate and then a little Lugol's solution are added to about 5 c.c. of the distillate, and the mixture is warmed. With large quantities of acetone, an immediate precipitation of yellow iodoform crystals (hexagonal plates or six-pointed stars) occurs (Fig. 8). When the amount of acetone is small (0.01 mg. or less), a few hours may be required for the formation of the crytsals, which are detected by microscopic examination of the sediment. Warming the tube intensifies the characteristic iodoform odor. The test is very delicate. Crystals may be demonstrable after twenty-four hours with as little as 0.0001 mg. of acetone. If the crystals are atypical, the precipitate is dissolved in alcohol-free ether and recrystallized.

Sources of Error.—Both alcohol and aldehyd give Lieben's test.

3. **Legal's Test.**—A few small crystals of sodium nitroprussid are dissolved in about 5 c.c. of the distillate. An excess of sodium or potassium hydrate is now added. If acetone is present, a red color develops, which soon changes to yellow. Glacial acetic acid, added in excess while the color is still red, causes a change to purplish red and finally to violet. The test indicates about 0.1 per cent of acetone.

Sources of Error.—According to v. Jaksch, paracresol gives a yellowish-red color with sodium nitroprussid and sodium hydrate; on adding an excess of glacial acetic acid the color changes to a rose red, and may be confused with the acetone reaction. Creatinin causes the same preliminary color changes as acetone, but on acidifying with glacial acetic acid the color gradually becomes green and then blue. When testing the distillate this difficulty is removed.

4. **Lange's Test.**[64]—About 15 c.c. of urine are placed in a test tube and treated with 0.5 to 1 c.c. of glacial acetic acid. After the addition of a few drops of a freshly prepared solution of sodium nitroprussid, ammonium hydrate is carefully layered above the urine. In the presence

[64] Lange, F. "Eine Ringprobe auf Azeton." *München. med. Wchnschr.* 1906, LIII, 1764.

of acetone an intense violet ring appears at the line of contact. The quantity of nitroprussid used is unimportant, but the amount added should not be enough to color the urine. The test, which is a modification of Legal's, is sensitive to acetone in 1/400 per cent solution. The reaction is not given by alcohol or aldehyd.

DIACETIC ACID

Diacetic acid or acetoacetic acid, the precursor of acetone, is usually found in urines which contain abnormal amounts of acetone. When urine is allowed to stand, the diacetic acid soon becomes converted into acetone, which in turn is lost within a few hours through volatilization or decomposition. Diacetic acid may, however, be kept in the urine with little loss for weeks by the addition of toluol to the specimen in a tightly stoppered bottle. Unless toluol be added, the urine should be tested for diacetic acid soon after it is voided.

Diacetic acid may be encountered in any of the conditions leading to acetonuria. The same is true regarding β-oxybutyric acid.

1. **Gerhardt's Test.**—To about 20 c.c. of urine in a test tube add an excess of 10 per cent ferric chlorid solution. If a precipitate forms, it is removed by filtration. To the filtrate more ferric chlorid is added, as long as it produces a perceptible darkening in color. A deep Bordeaux red color is produced by diacetic acid. The contents of the test tube are now halved, one portion being boiled, the other set aside as a control. If the color is due to diacetic acid, boiling for several minutes (two or more) lessens its intensity very perceptibly, owing to the breaking up of the diacetic acid.

The test indicates 0.04 to 0.05 per cent of diacetic acid.

Sources of Error.—After the administration of various drugs, notably salicylic acid, aspirin, diuretin, salol, phenacetin, acetates, formates, etc., a red color, at times indistinguishable from that produced by diacetic acid, may be seen. Except in the case of formates and acetates, the color *does not fade* after boiling a few minutes or standing several hours, as is the case when it is due to diacetic acid. When both diacetic acid and one of these drugs coexist, the urine is distilled, and the distillate tested for acetone. When the disturbing body is either a formate or an acetate, the urine is acidulated with sulphuric acid, cooled if necessary, and then extracted with an equal volume of ether. With a pipette the ether is transferred to another test tube and a small quantity of very

dilute watery ferric chlorid solution added; the tube is then well shaken. Diacetic acid causes a violet color in the watery layer, which changes to a Bordeaux red on the addition of more ferric chlorid. The color fades quickly on boiling the watery layer (remove ether first!). Formates and acetates do not give this reaction.

2. **Arnold's Test.**[65]

Reagents:

Solution A.—1 gm. of paramido-acetophenon is dissolved in 80 to 100 c.c. of distilled water with the aid of hydrochloric acid added drop by drop during vigorous shaking. Acid is added till the yellow solution becomes water clear. An excess of hydrochloric acid is to be avoided.

Solution B.—Sodium nitrate, 1 per cent aqueous solution.

The solutions keep well.

Method.—Two parts of solution A are mixed with 1 part of solution B (always prepare freshly at the time of making the test). Add an equal volume or less of the suspected urine and then 2 to 3 drops of strong ammonia, shaking well. All urines give a more or less intense brownish-red color. With excessive quantity of diacetic acid the addition of the ammonia produces an amorphous, brownish-red precipitate, but with smaller amounts no precipitate forms. A portion of the reddish fluid is placed in a wine glass or test tube, and a great excess of concentrated hydrochloric acid is added (to 1 c.c. of fluid add about 10 to 12 c.c. HCl). In the presence of diacetic acid the mixture takes on a beautiful purplish-violet color. With large amounts of diacetic acid the violet predominates, while with smaller quantities the red is more evident. With normal urine (free of diacetic acid) only a yellow color is obtained.

With small amounts of diacetic acid the reaction may fail if the urine be highly colored. In such case filter the urine through animal charcoal, and the reaction becomes positive with the water-clear filtrate. In using the filtrate add 2 to 3 parts of filtrate to 1 part of the mixed reagent.

The reaction is specific for diacetic acid and its ethyl ester. It is not given by drugs and is more delicate than Gerhardt's test (Arnold).

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[65] Arnold, V. "Eine neue Reaktion zum Nachweis der Acetessigsäure im Harn." *Wiener klin. Wchnschr.*, 1899, XII, 541.

β-OXYBUTYRIC ACID

β-oxybutyric acid, the third of the "acetone bodies," is found in the urine only in the presence of acetone or diacetic acid, or both, though the converse of this is not true. It occurs in largest amount in certain cases of diabetes mellitus. Its presence may be suspected when the urine is found to be definitely levorotatory after fermentation of the glucose; such a finding is not, of course, conclusive evidence of the presence of this body.

1. **Black's Test.**[66]—Five or 10 c.c. of urine are concentrated in an evaporating dish at a gentle heat to one-third or one-fourth of the original volume, which eliminates the acetacetic acid. The residue is then acidified with a few drops of concentrated hydrochloric acid, and made to a thick paste with plaster of Paris and allowed to stand until it begins to set. It is then stirred and broken up in the dish with a blunt stirring rod. The porous meal thus obtained is extracted twice with ether by stirring and decantation. The ether extract, which contains β-oxybutyric acid, is evaporated spontaneously or on the water bath. The residue is finally dissolved in water and neutralized with barium carbonate. The fluid is now poured into a test tube and treated with 2 or 3 drops of commercial hydrogen peroxid, the whole being mixed by shaking. The β-oxybutyric acid is oxidized to diacetic acid. Now add a few drops of 5 per cent ferric chlorid containing a trace of ferrous chlorid. On standing a few seconds a beautiful rose color develops, which slowly intensifies until it reaches a maximum, and then gradually fades, owing to the further oxidation of the acetacetic acid.

Sources of Error.—Black says that the chief precautions to be observed in carrying out the test are to be sure that the solution is cold and nearly neutral, and to avoid a large excess of hydrogen peroxid and iron. If too much of the oxidizing agents is added, and but little β-oxybutyric acid is present, the color developed is transitory or fails to appear. By starting with a small quantity and then adding more ferric chlorid at intervals of a few minutes, until no further color is produced, one is able to observe the full intensity of color, and thereby get a rough idea as to the amount of β-oxybutyric acid present.

The test is delicate. Black found that a solution containing 0.1 mg.

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[66] Black, O. F. "The detection and quantative determination of β-oxybutyric acid in the urine." *Jour. Biol. Chem.*, 1908, V, 207.

per cubic centimeter, or one part in 10,000, gave an easily recognized color.

2. **Hart's Test.**[67]—Hart adds to 20 c.c. of the suspected urine 20 c.c. of water and a few drops of acetic acid, and boils, until the volume is reduced to about 10 c.c. To this residue add water to the original volume, 20 c.c. Put this into two test tubes (*B* and *C*) of equal size, 10 c.c. in each test tube. To one of the test tubes (*C*) add 1 c.c. of peroxid of hydrogen, warm gently for about one minute (do not boil), and then allow the fluid to cool. Add to each test tube 0.5 c.c. of glacial acetic acid and a few drops of a freshly prepared solution of sodium nitroprussid, and mix. Overlay the solution in each test tube with 2 c.c. of ammonium hydroxid (Lange's test, p. 56). Allow the tubes to stand for four or five hours, and at the end of this time compare them. At the point of contact between the ammonia and the underlying fluid, *B* will show no ring (or a faint brown ring, if much creatinin is present); test tube *C*, to which the hydrogen peroxid was added, will show a purplish-red contact ring, if β-oxybutyric acid was originally present; if β-oxybutyric acid was not present, the two test tubes will not differ in appearance. If the two tubes are now shaken, the difference in color will be seen throughout the fluid; this difference is intensified by allowing the tubes to stand for fifteen or twenty minutes after shaking.

The oxidation of the β-oxybutyric acid to acetone by means of the hydrogen peroxid is said to be gradual, and reaches its maximum in about four or five hours, after which the color slowly fades. When a very large amount of β-oxybutyric acid is present, the difference in the two tubes may become evident in a few minutes. The two tubes should always be prepared as above. *B* will show whether all preformed acetone and diacetic acid have been driven off.

The presence of sugar does not interfere with the reaction. If albumin is present, it should be removed.

The method, though simpler than Black's, does not compare with the latter in delicacy. Hart finds that it will certainly detect β-oxybutyric acid when present to the extent of 0.3 per cent and probably less.

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[67] Hart, T. S. "The detection of β-oxybutyric acid in the urine." *Amer. Jour. Med. Sc.*, 1909, CXXXVII, 869.

UROBILINOGEN

Urobilinogen (see also arobilin) is normally present in the urine in traces. It is formed from bilirubin in the intestines through the reducing action of bacteria. With complete biliary obstruction, therefore, formation of urobilinogen ceases. It is converted into urobilin within a few hours after the urine is voided, so that it is necessary to employ fresh specimens in testing for it. In the twenty-four-hour specimen an excess of the chromogen, though originally present, may be missed by the time the examination is made; in this case urobilin may be looked for.

Ehrlich's Aldehyd Test.

Reagent:[68]

Dimethylparamidobenzaldehyd ..... 2.0 gm.
Hydrochloric acid (5 per cent)[69] .....100.0 c.c.

Dissolve. Keep in dark brown glass bottle.

About 10 c.c. of urine in a test tube are treated with a few drops of the reagent. In the presence of abnormally large amounts of urobilinogen a red color develops in the cold. In normal urine the red color appears only after heating. If the color fails to develop on heating, urobilinogen is absent.

UROBILIN

Urobilin, whose chromogen is urobilinogen, is a constituent of normal urine. Though it be lacking in the freshly voided specimen, traces of it are soon present, due to the action of light on urobilinogen. Large quantities of the pigment may impart a deep yellowish-brown color to the urine, though an excess may be present without noticeable change in the appearance of the specimen.

Urobilinuria (including *urobilinogenuria*) occurs:

1. In *congenital and acquired hemolytic jaundice.*
2. In *pernicious anemia,* during periods of active hemolysis.
3. In *malaria.*

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[68] As the reagent does not keep well, it should be prepared in small quantity, according to the demand.

[69] About 14 c.c. conc. HCl diluted to 100 c.c. with water.

4. In *hepatic disease,* especially cirrhosis and chronic passive congestion.
5. In *lead poisoning* at times.
6. In *acute infections,* especially pneumonia, scarlet fever, and acute rheumatic fever (probably due to an associated parenchymatous lesion of the liver).
7. In any condition in which there is *increased blood destruction,* as in several of the instances enumerated above, also after blood transfusion at times, after poisons which destroy the red corpuscles, such as benzol, nitrobenzol, pyrodin, etc.
8. After a large *internal hemorrhage.*
9. In *obstructive jaundice when complicated with cystitis.* In cases of obstructive jaundice, urobilin and urobilinogen are not found in the urine. Recently, however, in a patient in the Medical Clinic of the Cincinnati General Hospital with complete obstruction (acholic stools), Isaacs[70] demonstrated urobilinogen in the urine. There was a colon bacillus cystitis and bilateral pyelitis (diagnosis confirmed by autopsy). The urobilinogen was probably formed in the urinary bladder as a result of the reducing action of the bacteria on the bilirubin, as Isaacs pointed out.

1. **Spectroscopic Determination.**—When there is a considerable excess of urobilin it may be detected by direct spectroscopic examination of the urine. A small hand spectroscope is most convenient for the purpose. A few c.c. of clear urine, previously treated with a few drops of Lugol's solution (about 1 drop to 2 c.c.), and a few drops of mineral acid are examined directly with the spectroscope. The characteristic spectrum of acid urobilin, a single band (Fig. 9) between the green and the blue parts of the spectrum (between the lines *b* and *F* and extending a little to the right of *F* in the green), is seen. If the urine is very highly colored it may be necessary to dilute it before examining it with the spectroscope. On the other hand, with small amounts of urobilin the pigment should be extracted from the acidulated urine with amyl alcohol. The extract then presents the band of uroblin in acid solution.

The filtrate in the next two tests may also be examined spectro-

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[70] Case to be published by Dr. Raphael Isaacs.

Baumann, L. "The chemistry and clinical significance of urobilin." *Arch. Int. Med.,* 1921, XXVIII, 475.

scopically. It must be remembered that urobilin in alkaline solution shows a band between *b* and *F* which is nearer *b* than that seen in acid

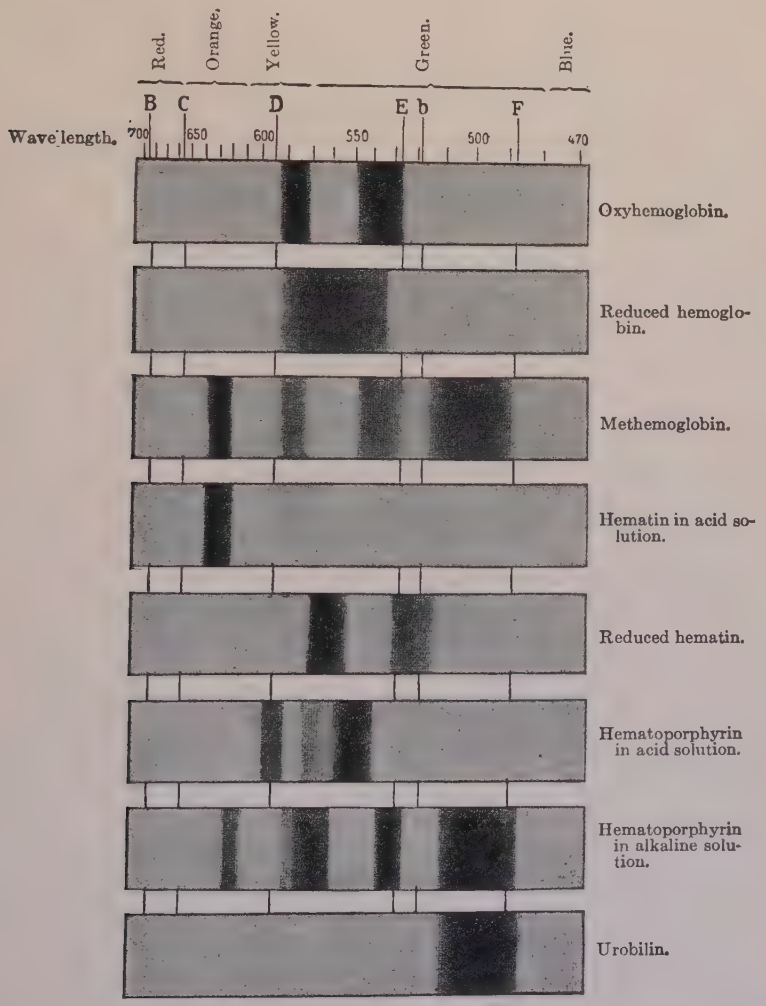

FIG. 9.—ABSORPTION SPECTRA (after Seifert and Müller).

solution. In solution with ammonia and zinc, the band is well seen. But if alkali be added to the urine for direct spectroscopic examination, fixed alkali will produce a darker band than ammonia.

2. **Schlesinger's Test.**[71]—This is the best test for routine work. It is delicate, easily performed, and requires no special apparatus.

About 10 c.c. of acid urine are treated with 5 or 6 drops of Lugol's solution to convert any urobilinogen present into urobilin. The urine is now mixed with an equal quantity of a saturated solution of zinc acetate in absolute alcohol and filtered. When held against a dark background and examined with transmitted light, the filtrate shows a beautiful green fluorescence, whose intensity is proportional to the quantity of urobilin present. The fluid may be examined spectroscopically.

The least trace of eosin or other fluorescing compound on the glassware may lead to misinterpretation of the result of the test.

The fluorescence may be made to appear more marked if the light be focused on the tube with a small hand lens.

The alcoholic zinc acetate solution precipitates other pigments, which may interfere with the reaction. However, when bilirubin is very abundant it is precipitated by adding 2 c.c. of 10 per cent calcium chlorid solution to 8 c.c. of urine. The mixture is filtered and the test applied to the filtrate. The test is sensitive to 0.002 per cent solutions of urobilin in urine, even in the presence of bile pigments.

3. **Jaffé's Test.**—The urine is treated with a few drops of Lugol's solution and then with an equal volume of 10 per cent alcoholic solution of zinc chlorid. The precipitate which forms is removed by filtration. The filtrate is rendered strongly alkaline with ammonia. A green fluorescence denotes the presence of urobilin. On spectroscopic examination the spectrum of urobilin in alkaline solution may also be observed. The single band is a little nearer *b* than that seen in acid solution (Fig. 9).

The test is not as delicate as Schlesinger's test. Zinc chlorid precipitates the interfering bodies less completely than zinc acetate, but alcoholic zinc chlorid is preferable to the aqueous solution, which is frequently recommended (Schlesinger).

When it is desired to determine whether urobilin is totally absent from the urine, large quantities of urine treated with Lugol's solution and mineral acid are extracted with a small volume of amyl alcohol, which may then be subjected to spectroscopic examination or to

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[71] Schlesinger, W. "Zum klinischen Nachweis des Urobilins." *Deutsche med. Wchnschr.*, 1903, XXIX, 561.

Schlesinger's test. The aldehyd test of Ehrlich is equally decisive in proving the absence of the pigment if a perfectly fresh specimen of urine be employed.

BILE PIGMENTS

Bile pigments occur in the urine in *patients with jaundice, excepting* only cases of congenital and acquired hemolytic jaundice; in these two instances, the urine is laden with urobilinogen and urobilin, but contains no bile.

Bile pigments, never normally present in the urine, may or may not cause an appreciable alteration in its appearance, the result depending on the concentration of the coloring matter. When much is present the urine has a dark brown, at times a greenish-brown, color. The pigments exist in the urine as such or as soluble combinations with the alkalies or alkaline phosphates. Thus it happens that *bilirubin (hematoidin) in crystalline form* is not usually seen in the urine; but in the urine of infants with small content in phosphates such crystals not infrequently form. They appear as yellowish or brownish-red needles or as rhombic plates or prisms, the latter often with rounded angles. The crystals are insoluble in water. In chloroform, especially if hot, they dissolve readily (1:600), imparting their color to the solution. In dimethylanilin, bilirubin crystals are very soluble (1:100). The alkaline compounds of bilirubin found in the urine are insoluble in chloroform. Bilirubin may, therefore, be removed from chloroform solution by alkali. The bilirubin as found in the urine is precipitated by the addition of hydrochloric acid. From ammoniacal solution bilirubin is precipitated by barium chlorid, lead acetate, and silver nitrate.

Solutions of bilirubin possess no characteristic absorption bands; there is a continuous absorption from the red to the violet end of the spectrum.

Qualitative Tests

1. **Foam Test.**—The urine is shaken vigorously and, if it contains considerable bile pigment, the foam presents a distinct yellow color. The test is practically specific, but is not very delicate. Similarly, icteric urines show a *yellow staining of the sediment*, and a similar color is left on filter paper, through which such urines have been passed.

2. **Gmelin's Test.**—The acid urine is superimposed carefully on yellow nitric acid [72] in a test tube; the layering of the two fluids must be sharp. In the presence of bilirubin a play of colors is observed at the line of contact of the two fluids. The colors from above downward are green, blue, violet, red, yellow. A piece of white paper, held behind the test tube, with the light at the examiner's back, aids in the recognition of the colors. The green color is the most important. The reaction is said to be positive in a dilution of 1:80,000.

Albumin and urobilin do not interfere with the reaction. Much indican may lead to confusion at times; other tests should then be resorted to.

3. **Rosenbach's Modification of Gmelin's Test.**—The urine, slightly acidified with hydrochloric acid, is passed through a small filter paper several times. The paper is then unfolded and blotted lightly with dry paper. The stain on the paper is now touched with a drop of yellow nitric acid, when, in the presence of bile pigment, a play of colors is seen at the edge of the drop. From within outward the colors are yellow, red, violet, blue, and green. The green color, the result of oxidation of bilirubin to biliverdin, is again the most important color; in its absence the test is negative. If the paper be allowed to dry, it should be moistened with a drop or two of water before applying the acid. The test is very delicate and practically specific.

4. **Trousseau's Test.**—The urine, if alkaline, is acidified with acetic acid. Tincture of iodin, preferably diluted 1:10 with alcohol, is superimposed on the urine. In the presence of bile, a green ring forms at the line of contact within about a minute. The test is very delicate.

5. **Huppert's Test.**—In deeply pigmented urines or in those rich in indican or hemoglobin, this test is preferable to Gmelin's. The urine is made alkaline with sodium or ammonium carbonate, and then calcium chlorid solution is added as long as a precipitate forms. The mixture is passed through a small filter, the precipitate washed with water, and the precipitate and filter paper transferred to a test tube or porcelain evaporating dish. Acid alcohol (concentrated hydrochloric acid, 5 c.c., alcohol, 95 c.c.) is now added and carefully heated to boiling. In the presence of bilirubin the color of the alcoholic solution changes to green

[72] Yellow nitric acid is quickly obtained by adding a small piece of pine or other soft wood to nitric acid. In a short time, nitrous acid, HNO_2, is evolved. The acid should be light yellow; too much nitrous acid may accelerate the oxidation to such an extent that the colors are missed entirely.

or blue. The delicacy of the reaction varies between a dilution of bile of 1:500,000 and 1:1,000,000 (Hammarsten).

6. **Hammarsten's Test.**

Reagent:

Nitric acid (25 per cent)	1 part
Hydrochloric acid (25 per cent)	19 parts

The reagent may be kept for about a year. It is not ready for use until its color becomes yellow.

Ordinarily it is sufficient to pour a few drops of urine into 2 or 3 c.c. of the reagent. Almost immediately after shaking, the mixture takes on a green or bluish-green color, which will persist about twenty-four hours. When only traces of bile pigment are present, 10 c.c. of the acid or neutral (not alkaline) urine are treated with a 10 per cent solution of barium chlorid, which is added as long as a precipitate forms. The mixture is now centrifugalized. The supernatant fluid is poured off and the sediment is shaken with about 1 c.c. of the reagent and again centrifugalized. The supernatant fluid is now a beautiful green, which changes upon further addition of the reagent through blue to violet, red, and finally reddish-yellow. The green color is obtained in the presence of 1 part of bile pigment to 500,000 to 1,000,000 parts of urine. In the presence of considerable amounts of blood-coloring matter or other pigments, a 10 per cent solution of calcium chlorid should be substituted for the barium chlorid solution.

BILE ACIDS

The salts of glycocholic and taurocholic acids may be found in the urine, *not infrequently in the absence of bile pigments,* as shown by Symmes Oliver.[73] When the urine contains an increase in these salts, it is cloudy, if the reaction is acid; and the turbidity is increased, if acid is added to the urine. The salts remain in solution in neutral or alkaline urines. It is important to remember, too, that the salts may be precipitated by the addition of acetic or nitric acids to the urine, even in the cold, and that they form a ring just above the line of contact in Heller's nitric acid test for albumin. These facts lead not infrequently to error, since an albuminuria may be reported, when in reality the supposed albumin reactions are those of bile salts (Oliver).

[73] Oliver, S. F. "The effect of bile salts in the urine on routine tests for albumin." *Jour. Lab. and Clin. Med.,* 1922, VII, 743.

Bile acids are increased in the urine, according to Oliver, in:

1. *Obstructive jaundice.*
2. *Cirrhosis of the liver,* particularly the hypertrophic forms.
3. *Pernicious anemia.*

Qualitative Tests

1. **Hay's Test.**—Bile salts, like soaps, have the property of lowering the surface tension of a liquid. This fact is taken advantage of in Hay's test. A small amount of finely powdered flowers of sulphur is sprinkled on the surface of the cool urine. In the presence of bile salts, the sulphur sinks at once, or within a minute after shaking gently. The test is delicate but is not specific.[74]

2. **Peptone Test.**—Prepare an aqueous solution of Witte's peptone, about 1 per cent. Filter, and to about 5 c.c. of this reagent in a test tube add the urine to be examined drop by drop. A white precipitate forms, if bile salts are present. Previous to testing, the urine should be diluted, if necessary, to a specific gravity of 1.008, filtered if there is a turbidity, and rendered faintly acid with acetic acid (Oliver).

3. **Egg Albumen Test.**—Prepare a 2 per cent aqueous solution of egg albumen, weakly acidified with acetic acid and preserved with a small amount of salicylic acid. The technic of the test is the same as that of the peptone test. A milkiness of the solution indicates the presence of bile salts (Oliver).

4. **Starch Test.**—The reagent is prepared by adding 2 gm. starch to 100 c.c. of distilled water; this is slightly acidified with dilute acetic acid and preserved by the addition of a small amount of salicylic acid; it is not boiled. The test is carried out in the same way as the peptone and egg albumen tests (Oliver).

Tests 2, 3, and 4 indicate bile salts in dilutions as high as 1:25,000. Hay's test is said to be more delicate.

HEMATOPORPHYRIN

Hematoporphyrin, an iron-free derivative of hemoglobin, is a normal urinary pigment occurring in traces. When urine contains hematoporphyrin in considerable concentration, its color is usually dark red.

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[74] Mathews, A. P. *Physiological Chemistry.* N. Y., 1921, p. 986.

Hematoporphyrinuria has been met with:

1. In *dilatation of the duodenum.*
2. In *lead poisoning.*
3. After *prolonged administration of trional and sulphonal.*
4. In the *acute infections,* at times.
5. In some cases of *Addison's disease.*
6. In *tuberculosis* at times.
7. In *cirrhosis of the liver.*

Garrod's Test.—One hundred c.c. of urine are treated with 20 c.c. of 10 per cent sodium hydrate; this precipitates the phosphates, which carry the pigment down with them. The precipitate is collected by filtration or centrifugalization, and is dissolved in acid alcohol (HCl, 5 c.c., alcohol, 95 c.c.). The solution is examined spectroscopically for the bands of hematoporphyrin in acid solution (Fig. 9), one just to the left of *D,* the other—a broader band—between *D* and *E.* The result may be further controlled by obtaining the absorption bands of hematoporphyrin in alkaline solution. The alcoholic solution is rendered alkaline with ammonia, acetic acid is added till the precipitate of phosphates is dissolved, and the pigment is then extracted with chloroform. The latter is examined spectroscopically for the four bands of hematoporphyrin in alkaline solution (Fig. 9); the first about midway between *C* and *D,* the second at *D* extending to the right of it, the third at the left of *E,* the fourth—a broad band—beginning at *b* and extending almost to *F.*

In place of sodium hydrate, Salkowski recommends that the urine be treated with a solution composed of equal parts of cold saturated barium hydrate and 10 per cent barium chlorid, while Hammarsten prefers a solution of barium acetate. In either case the precipitate is washed and then dissolved in acid alcohol, as in Garrod's test, and the alcoholic solution is examined for the spectrum of hematoporphyrin in acid solution. By adding an excess of ammonia the bands of alkaline hematoporphyrin are obtained.

In certain instances the urine contains hematoporphyrin in such concentration that direct spectroscopic examination reveals its presence. In such case the alkaline spectrum is the one usually observed, though the spectrum of hematoporphyrin in acid solution may be seen, and is still sharper after acidifying the urine with a mineral acid.

HEMOGLOBIN

Hemoglobin in the urine is always pathological. The hemoglobin is often changed to methemoglobin. If the urine contains hemoglobin in relatively high concentration, it assumes a dark red color, which is imparted to the sediment. The latter, however, is often dark brown. Occasionally crystals of hematoidin are found on examination of the sediment (see bilirubin crystals); when placed in a porcelain dish with a drop of yellow nitric acid, a play of colors, especially a green ring, is seen at the periphery of the drop (Gmelin's reaction). In hemoglobinuria red blood corpuscles are usually absent or present in small number.

Hemoglobinuria is present:

1. In *paroxysmal hemoglobinuria.*
2. In *blackwater fever.*
3. At times in the *severe forms of the acute infectious fevers* (scarlatina, syphilis, typhus, malaria, yellow fever).
4. After exposure to *cold,* at times.
5. After *severe burns.*
6. After large *internal hemorrhages* at times.
7. After *certain poisons,* such as nitrobenzol, pyrodin, chlorates, sulphonal, carbon monoxid, etc.
8. In *pernicious anemia* (rare).
9. After *blood transfusion* (rare).

1. **Spectroscopic Determination** (Fig. 9).—The urine, if neutral or alkaline, is rendered slightly acid with dilute acetic acid, and is filtered till perfectly clear. If very highly colored it may be necessary to dilute the specimen before examining it spectroscopically.

a. Oxyhemoglobin is characterized by the appearance of two bands between *D* and *E,* the narrower band being near *D.*

b. Reduced hemoglobin is recognized by a single broad band, extending from *D* toward *E.*

c. Methemoglobin in neutral or weakly acid solution produces a dark band in the spectrum between *C* and *D,* near *C.* The two additional bands seen between *D* and *E* are usually attributed to the coexistence of oxyhemoglobin in the solution. In alkaline solution methemoglobin presents two absorption bands between *D* and *E,* resembling those of

oxyhemoglobin, except for the fact that the narrower band in this case is situated at the right.

d. Hematin is found in the urine very infrequently. In acid solution its spectrum resembles closely that of methemoglobin in neutral or acid solution, consisting of a single dark band in the red, extending to the right of *C*. Hematin is readily differentiated from methemoglobin by the fact that the addition of ammonia and a reducing substance, such as ammonium sulphid, to the acid solution converts the spectrum into that of hemochromogen.

e. Hemochromogen [reduced hematin (*d*)] presents two dark bands, one about midway between *D* and *E*, the other to the right of *E*. Both bands are nearer the green end of the spectrum than those of oxyhemoglobin.

In case the urine is very deeply pigmented, the *spectroscopic examination* is facilitated by dilution with water, since concentrated solutions absorb the spectrum diffusely. On the other hand, with small amounts of hemoglobin, the delicacy of the spectroscopic test depends very largely upon the thickness of the layer of urine, through which the light passes to the spectroscope. Schumm [75] finds that with the usual test tube hemoglobin may be detected spectroscopically in a dilution of 1:2,000, whereas, if the urine be placed in the polariscope tube 10 or 20 cm. long, it is possible to recognize one part of hemoglobin in about 25,000 parts of urine. Roughly, this is equivalent to one drop of blood in the twenty-four-hour specimen. If the oxyhemoglobin has been changed to methemoglobin, Schumm recommends the following procedure: 50 c.c. of urine, 5 c.c. of glacial acetic acid, and 40 to 50 c.c. of ether are shaken together. The ether, after it has separated, is drawn off and shaken with 5 c.c. of water, which is then removed. The guaiac test (p. 72) is then applied to a part of the ether extract. To the remainder add ammonia in excess (keep the mixture cool) and shake well. The ammonia layer and a part of the ether are allowed to run into a glass, ammonium sulphid is added, and the bands of alkaline hemochromogen are looked for.

The following tests are applicable alike to the detection of *hemoglobinuria* and *hematuria* (see p. 96).

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[75] Schumm, O. "Untersuchungen über den Nachweis von Blut im Harn mit Hilfe des spektroskopischen und einiger spektroskopisch-chemischer Verfahren." *München. med. Wchnschr.*, 1908, LV, 1488.

2. **The Guaiac Test.**[76]

Reagents:

Guaiac resin, powdered.
Alcohol.
Hydrogen peroxid *or* ozonized oil of turpentine.

Tincture of guaiac is freshly prepared at the time of making the test by adding a knife point of powdered guaiac to about 5 c.c. of alcohol, shaking till solution occurs.

Equal parts of tincture of guaiac and hydrogen peroxid are mixed and are then layered above the urine by inclining the test tube and pouring the mixture in very slowly. The urine, if neutral or alkaline, is acidified with acetic acid before testing. An opaque ring forms at the line of contact between the fluids; gradually a distinct blue color develops.

The test is very delicate, but it is not specific for blood. Disturbing substances are much less apt to be encountered in the urine than in the stools or gastric contents. Pus, when present, gives the blue color, but the reaction occurs without the addition of the peroxid. The urine may be treated with glacial acetic acid and extracted with ether (for details consult p. 159). For a complete list of the disturbing substances the reader is referred to the monograph of Kastle.

This test is very delicate; it may be positive with 1 part of blood in 20,000 to 40,000 parts of urine. It is chiefly of value when negative. A positive test does not mean the presence of blood—it must be confirmed by other tests; but a negative reaction is conclusive evidence of the absence of blood. The activity of the guaiac should be proved occasionally.

Several other chromogenic substances have been used successfully in place of guaiac, but they are all open to the same objection, i. e., lack of specificity. *Benzidin, aloin,* and *phenolphthalin* are the bodies most frequently substituted for guaiac.

3. **Heller's Test.**—The urine, if alkaline, is rendered neutral or slightly acid with acetic acid, and is then boiled. If much blood is present, a precipitate of albumin and hematin forms. The hot urine is now treated with sodium or potassium hydrate. The phosphates are precipitated and carry down with them any hematin present. The latter colors the precipitate red, which constitutes a positive reaction. In case

[76] For a full discussion of this and allied tests, see Kastle, J. H. "Chemical tests for blood." *Bull. No. 51,* U. S. Pub. Health & Mar. Hosp. Serv., Wash., pp. 1-62, 1909.

the phosphates have already been precipitated from the urine, normal urine may be added to supply the salts in solution; or a little calcium chlorid solution is added to the urine, which is then boiled, and sodium phosphate is poured into it with the sodium hydrate (Hammarsten).

To prove beyond question that the red precipitate is caused by blood pigment, the precipitate is subjected to Teichmann's hemin crystal test.

Heller's test is not very delicate, and is, therefore, less used now than formerly.

4. **Teichmann's Hemin Crystal Test.**—The precipitate obtained in Heller's test or from treating the urine with tannic acid is used for this test. The excess of phosphates may be removed by washing the precipitate with very dilute acetic acid. The precipitate is then dried on the filter paper, and a small amount of it transferred to a clean glass slide. To it add a few *small* crystals of sodium chlorid. Crush the crystals and mix the powder with the precipitate. A cover glass is placed on the material, and glacial acetic acid is run under it. Heat the preparation just short of boiling ¾ to 1 minute, replenishing the acid as necessary. The fluid turns brown. The specimen is allowed to cool a few minutes, and is then examined microscopically for the brown rhombic plates of hemin (Fig. 10). It is often necessary to reheat the specimen several times before the crystals are obtained. Instead of heating the specimen, it may be set aside for twenty-four hours before examining it; in this case the crystals are usually somewhat larger. With small crystals, high magnification may be required for their recognition.

Fig. 10.—Teichmann's Hemin Crystals.

The test is specific for hemoglobin. It often fails if too much sodium chlorid is added, or if the specimen is overheated.

MERCURY IN THE URINE

In cases of poisoning with bichlorid of mercury, the urine and feces or gastric contents should be tested for mercury. The following technic for the detection of mercury in the urine has been proposed by Vogel and Lee [77] and has been employed by Weiss [78] in a large series of cases in the Cincinnati General Hospital. The test is simple and is specific.

[77] Vogel, K. M. and Lee, O. I. "Detection of mercury in the excretions." *Jour. A. M. A.*, 1914, LXII, 532.

[78] Weiss, H. B. "A method of treatment of mercuric chloride poisoning." *Jour. A. M. A.*, 1917, LXVIII, 1618; "The principles of treatment in mercuric chloride poisoning." *Jour. A. M. A.*, 1918, LXXI, 1045.

Method of Vogel and Lee.—To about 150 c.c. of urine, add 5 c.c. of concentrated hydrochloric acid, and evaporate over a free flame till the bulk has been reduced to about 25 or 30 c.c. Next add about 2 c.c. of hydrochloric acid to replace the loss by evaporation, and about 2 gm. of potassium chlorate to oxidize the organic material present. When this is accomplished, the fluid becomes pale yellow or colorless. It is then diluted to about 60 c.c. and is boiled vigorously until the chlorine gas previously evolved has been driven off, which is shown by the absence of chlorine odor from the steam. The solution usually darkens again on cooling. A piece of copper wire (about 18 gauge) about 4 cm. in length, bent back on itself twice and cleansed by boiling a short time in dilute hydrochloric acid, is dropped into the solution and allowed to remain for an hour or more. If considerable amounts of mercury are present, it will be found to be coated with a silvery film of metallic mercury; but this is not sufficient to establish the identity of the metal, and if it exists only in traces, the changes in the appearance of the wire may be inconclusive. The wire is therefore removed from the dish with a glass rod, is washed with a little water and is gently dried by rolling it on a piece of filter-paper, pains being taken to avoid unnecessary handling. It is then allowed to slip to the bottom of a glass tube from 3 to 5 mm. in diameter and 15 cm. in length, which is sealed at one end, and a cylindrical plug of gold-leaf, such as dentists use, is pushed into the tube until it is within 2 cm. of the wire. Holding the tube horizontally, the end containing the wire is gradually heated by brief successive introductions into the small flame of a Bunsen burner or spirit lamp, care being taken to avoid heating the part of the tube containing the gold-leaf. The latter must be examined frequently for any change of color, especially the end of the gold toward the wire. If mercury is present, it will manifest itself by the appearance of a silvery patch of amalgam in this situation. If the amount of the metal is exceedingly minute, there will be simply a pale discoloration of the gold, seen better with a hand lens or by removing the gold-leaf from the tube and examining it with the low power of the microscope.

The authors further state that if the gold-leaf is too close to the wire, the heat traveling along the tube may cause the deposit of mercury which at first was distinctly visible, to become diffused so that it grows less evident. In doubtful cases the experiment should be repeated, using larger quantities of urine and allowing the wire to remain longer in the solution. If a positive result is obtained, the tube may be sealed and

kept as a permanent record. If further confirmation of the identity of the mercury is required, the gold-foil may be suspended in a tube containing a few crystals of iodin, which are then warmed very gently. The mercury thus becomes converted into the red mercuric iodid; or if the amount is considerable, the metal may be distilled by heating the gold-foil in the tube and looking with the lens for a deposit of very minute droplets of metallic mercury in the cooler parts of the tube.

The test is very delicate. One mg. of mercury in 100 c.c., equaling a dilution of 1:100,000, may be detected in the urine, stomach contents and stools, the wire being allowed to remain in the fluid two hours; the amount of amalgam formed on the gold is sufficiently large to be recognized with the unaided eye. A positive test is obtained at times after administration of 2 grains of calomel. It is, therefore, desirable, before making a diagnosis of mercury poisoning, to find out whether or not the patient has been given calomel.

The quantity of mercury which the copper is capable of abstracting from the fluid is a function not only of the concentration of the solution, but also of the time of contact, so that if the test is negative after the wire has been immersed for one or two hours, another piece of wire should be dropped into the fluid and allowed to remain over night, if necessary.

THE DIAZO REACTION

Ehrlich's diazo reaction is never given by normal urine, but is of frequent occurrence in febrile diseases, comparatively rare in afebrile conditions.

Diazo bodies may be present in the urine:

1. In *typhoid* and *paratyphoid fevers* in the great majority of the cases, at times as early as the third or fourth day of the disease.
2. In the majority of the cases of *measles.*
3. In *miliary tuberculosis* and in *advanced pulmonary tuberculosis,* usually considered a sign of bad prognosis.

   A positive test may occur, but is *less common:*
4. In *pneumonia, scarlet fever, diphtheria,* and *erysipelas.*
5. In *meningitis* and *acute rheumatic fever,* in *malignant disease,* and following *typhoid vaccination.*[79] a positive test is rare.
6. After administration of *atophan,*[80] a positive test has been reported.

[79] Svestka, V. "Die Urochromogen-Reaktion Weiss im Harne bei Typhus abdominalis." *Wiener klin. Wchnschr.*, 1915, XXVIII, 1054.

[80] Skorczewski, W. and Sohn, I. "Ueber einige im Atophanharne auftretende charakteristische Reaktionen." *Wiener klin. Wchnschr.*, 1911, XXIV, 1700.

Reagents:

Solution 1:

Sodium nitrite	0.5 gm.
Distilled water	100.0 c.c.

Dissolve. The solution does not keep well.

Solution 2:

Sulphanilic acid	5.0 gm.
Hydrochloric acid, conc.	50.0 c.c.
Distilled water to	1,000.0 c.c.

Dissolve.

One part of solution 1 is mixed with 50 parts of solution 2. The mixture should be prepared freshly each day, as it is not permanent longer than twenty-four hours.

Equal quantities of the mixed solutions and the urine are mixed in a test tube. Ammonium hydrate is then run down the side of the tube, which is inclined, so that it forms a layer at the top. A red ring is formed at the line of contact of the fluids. The test tube is sealed and shaken vigorously. If the foam is colored pink, the reaction is positive. The color fades rather rapidly.

It is quite possible to misinterpret the result of the test, since a salmon or yellowish-red or brownish-red foam is not infrequently observed. It is essential to discard all results as negative unless the foam is unquestionably pink.

At times the bodies causing the diazo reaction exist in the urine in such dilution that a positive reaction is not obtained. In such cases the test again becomes positive, if the urine is concentrated to a small volume on a water bath.

If the sodium nitrite solution is used in greater strength than 1:50, normal urine may give the color reaction. After the administration of atophan (phenyl-quinolin-carboxylic acid), 3 gm. daily, a positive diazo test may be given by normal urine.[81]

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[81] Skorczewski, W., and Sohn, I. *Loc. cit.*, p. 75.

CHYLURIA

The admixture of chyle causes the urine to appear more or less milky, depending partly on the proportion of chyle, but still more on its fat content. Whether parasitic (filaria) or non-parasitic in origin, chyluria is probably always due to a direct anatomical communication between the lymph channels and the genito-urinary tract, as Magnus-Levy [82] and others have pointed out. Ureteral catheterization often reveals the fact that the chyluria is unilateral. The symptom is intermittent, as a rule, depending on the posture of the patient; in some cases it appears during the day, in others only at night.

Chyluria is uncommon in this country. Two classes of cases are generally recognized:

1. *Parasitic.* This is generally due to *filariasis.* Some cases of *schistosomic origin* have been reported. Two cases have been reported, due to *hydatids,* with hydatid membrane in the urine. Quarelli has recorded a case of *malarial chyluria,* associated with malarial parasites in the blood, and cured by quinine (Castellani and Chalmers).
2. *Non-Parasitic.*

In two-thirds of all cases, the chyluria disappears either in the reclining or the upright posture, and, in both the parasitic and the non-parasitic varieties, the phenomenon is due to a direct communication between the urinary and lymphatic channels (Magnus-Levy).

All of the normal ingredients of chyle may be found in the urine. They are:

1. *Neutral Fat.*—Droplets of neutral fat (Fig. 13) are always present; they are derived from the chyle, not from the blood, and the quantity found varies directly with that ingested in the food. The droplets vary considerably in size; some are so small that they are only seen distinctly with the oil immersion. They possess a sharp contour and are highly refractive. The addition of a drop of Sudan III or of Scharlach R (saturated solution in 70 per cent alcohol) stains the fat droplets an orange or reddish-yellow color. From the alkaline urine the fat may be extracted by means of the usual fat solvents, such as ether.

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[82] Magnus-Levy, A. "Ueber europäische Chylurie." *Ztschr. f. klin. Med.,* 1908, LXVI, 482.

2. *Cholesterin* (Fig. 13) and *lecithin* are found if large quantities of urine are extracted with ether. Their quantity is dependent largely on the food.

3. *Sugar* may or may not be discovered in the urine. It has been shown that chyle contains about 0.1 per cent sugar in hunger or on a fat or protein diet. One part of chyle in two parts of urine under these circumstances would give about 0.03 per cent glucose—too little to detect with the usual clinical tests. On the other hand, after a large carbohydrate meal, especially a meal containing an excess of sugar, the urine may contain 0.3 to 0.4 per cent of glucose. With this amount glycosuria is easily recognized.

4. *Lymphocytes* are always to be seen, either in the sediment of the centrifugalized specimen or caught in the meshes of the clots, which occasionally form in the urine.

5. *Albumin* is generally found in the urine, unless the proportion of chyle be very small. With appropriate tests, such as fractional precipitation of the urine with ammonium sulphate, globulin and fibrinogen may usually be demonstrated.

6. *Filaria Bancrofti.*—In parasitic chyluria the embryos of *Filaria bancrofti* are present in the sediment or in the clot (see pp. 110 and 350).

LIPURIA

Small quantities of fat are not unusual in the urine. When epithelial or pus cells are present it is common to find fat droplets in them, and a few droplets are found free in the urine. The term lipuria is reserved for those conditions in which the fat is so abundant that it is recognized macroscopically, and is usually associated with a lipemia. It is important to exclude fat from external sources, such as dirty containers for the urine, the lubricant on catheters, the willful admixture of fat or milk for purposes of deception, etc. Fat is recognized by its appearance and microchemical reactions (p. 77). Unless it is present in the form of an emulsion, it is seen on the surface of the fluid. In the absence of chyluria, lipuria generally indicates an excess of fats in the blood—lipemia.

FERMENTS IN THE URINE

A number of enzymes have been discovered in the urine—pepsin, trypsin, lipase, diastase, etc. From a diagnostic standpoint diastase appears to be the most important, though the determination of lipase has also been of some value in pancreatic disease.

1. **Wohlgemuth's Method for the Determination of Diastase.**[83]—A 1 per cent starch solution is prepared. Merck's or Kahlbaum's soluble starch is employed. The starch powder is stirred in cold distilled water, which is then heated about 10 minutes with constant stirring, until the solution is clear. It is cooled and is then ready for use. Into each of several test tubes 5 c.c. of the starch solution are placed with a pipette. Next add varying fractions of 1 c.c. of urine to the tubes, which have been numbered, beginning with 0.2 c.c. and decreasing gradually. Add a small quantity of toluol to each tube to prevent bacterial growth, and place the tubes in the incubator at 37° C. for twenty-four hours. The tubes are then removed from the incubator and filled almost completely with ice water. To each tube add 1 drop of $\frac{N}{10}$ iodin solution, mix well, and observe for the blue color of the starch-iodin reaction. The first tube which shows no blue is selected. From the known proportions of urine and starch solution in this tube, calculate the number of cubic centimeters of 1 per cent starch solution which 1 c.c. of urine would convert to dextrin and sugar. Assuming the result to be 150, it is expressed as follows: $D\,\frac{37^{\circ}}{24h} = 150$. This means that the urine examined contained sufficient diastase (D) to convert 150 c.c. of 1 per cent starch solution to dextrin and sugar, acting at 37° C. for 24 hours.

Wohlgemuth employs the urine obtained at the second voiding in the morning for examination. The diastase is greatest in the urine during fasting, and decreases three to four hours after meals. The highest normal value which he has obtained for the urine is 156.

2. **Determination of Lipase According to Hewlett.**[84]—Hewlett has adapted the ethyl butyrate method of Kastle and Loevenhart to the determination of lipase in the urine. The procedure follows, in the

[83] Wohlgemuth, J. (a) "Ueber eine neue Methode zur quantitativen Bestimmung des diastatischen Ferments." *Biochem. Ztschr.*, 1908, IX, 1. (b) "Untersuchungen ueber die Diastasen. Beitrag zum Verhalten der Diastase im Urin." *Ibid.*, 1909, XXI, 432. (c) "Beitrag zur funktionellen Diagnostik des Pankreas." *Berlin. klin. Wchnschr.*, 1910, XLVII, 92.

[84] Hewlett, A. W. "On the occurrence of lipase in the urine as a result of experimental pancreatic disease." *Jour. Med. Research*, 1904, XI, 377.

author's words: Five c.c. of urine are placed in each of three flasks. The urine in the second flask is then boiled. To the urine in the third flask are then added three drops of a 1 per cent solution of phenolphthalein and tenth normal sodium hydrate is allowed to run in from a burette, until a faint pink color appears throughout the fluid. The amount of sodium hydrate used is read off, and a like amount is added to the first and second flasks. To each of these two flasks, the first of unboiled urine, the second of boiled urine, are then added 0.25 c.c. of ethyl butyrate and 0.1 c.c. of toluene, and they are placed in a thermostat at 38° C. for about 20 hours. The toluene is added to prevent the growth of bacteria. At the end of this time each flask is taken out, and sufficient tenth normal hydrochloric acid is added to more than neutralize the alkali previously added by 0.5 c.c. Each specimen is then shaken in a separating funnel with 50 c.c. of redistilled ether, and the ether is separated. After adding three drops of a 1 per cent solution of phenolphthalein to 25 c.c. of pure alcohol, the latter is brought to the neutral point. The ether extract from the separating funnel is now added to the neutralized alcohol, and its acidity is determined by titrating with N-20 potassium hydrate solution (alcoholic). Any decided difference between the acidity of the ethereal extracts of the boiled and of the unboiled urine is due to the butyric acid formed by the cleavage of the ethyl butyrate; and, where the difference in acidity is at all great, the odor of butyric acid can be recognized.

In *normal* healthy men (and dogs) the urine contains merely traces of lipase. The greatest difference found by Hewlett was 0.35 c.c. of twentieth normal potassium hydrate—usually 0.1 or 0.2 c.c.

THE URINARY SEDIMENTS

The sediment may be obtained for microscopic examination by allowing it to settle in a conical specimen glass [85] or, preferably, by centrifugalizing the urine. The objection to sedimentation is that certain of the formed elements, especially casts, may be more or less completely digested, if the specimen be allowed to stand too long, whereas with a centrifuge

[85] The most satisfactory sedimenting glass with which the writer is familiar is one designed by Dr. J. S. Brotherhood (see Fig. 11). It permits one to take the specific gravity without transferring the urine to another receptacle, and its shape insures concentration of the sediment at the bottom. The weight of the base is an advantage, as the glass is not easily upset. It may be obtained from the Arthur H. Thomas Co., Philadelphia.

the examination may be made almost as soon as the urine is passed. It is particularly in alkaline urines that casts rapidly disappear. It is very important that a perfectly *fresh specimen* of urine be employed for microscopic examination. With urine which has stood for twenty-four hours it is often impossible to gain a correct impression of the sediment. Crystals, which were not present when the urine was voided, may have formed, while the more important organized material may have become so altered that it is no longer recognizable.

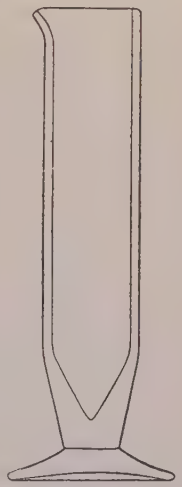

FIG. 11.—THE SYDENHAM SEDIMENTING GLASS.

The sediment is removed with a pipette and several drops of it are transferred to a glass slide. A cover glass should *not* be placed on the specimen for the preliminary examination, though it may be required later. When the sediment is scanty the few drops of urine adhering to the outer surface of the pipette should be wiped off to prevent dilution of the specimen. On the other hand, with abundant sediment it is often advantageous to thin it somewhat so that the various elements are separated and their recognition made less difficult.

The preliminary examination of a urinary sediment[86] should always be made *with low magnification and with the light cut off as much as possible.* Usually this examination is sufficient. But, if all the elements in the preparation cannot be recognized in this way, a cover glass is placed on the drop of sediment, which is now examined under higher magnification. The oil immersion lens is not employed with a wet specimen, nor is it necessary.

For microchemical tests a cover glass is placed on a drop of the sediment, and any excess of moisture removed with a blotter. With a pipette a drop of the reagent is placed on the slide at one side of the cover glass, while a piece of blotting paper is touched to the opposite side. The absorption of fluid by the paper creates a current, which draws the reagent under the cover glass. The effect upon the sediment is observed with the low power objective. In case it is necessary to use the higher power, great care should be exercised that the lens escapes the reagent.

[86] H. Rieder's "Atlas der klinischen Mikroscopie des Harnes" (Leipzig, 1898) is an extremely valuable and useful reference work on urinary sediments.

The Unorganized Sediments

For convenience the unorganized sediments are divided into those which occur chiefly in acid urine, and those which are encountered mainly when the reaction is alkaline. It must be remembered that the classification is by no means absolute; it frequently happens that a deposit, usually found in an acid urine, persists after the reaction has become alkaline, and, again, that a sediment which is generally met with in alkaline urine may make its appearance while the reaction is still acid.

Sediments in Acid Urine

1. **The Quadriurates of Sodium and Potassium.**—The quadriurates of sodium and potassium, the *amorphous urates,* are chiefly responsible for the pink, salmon-colored, yellow, or reddish deposits which may be seen in an acid urine. The salts are especially apt to be precipitated from concentrated specimens as they become cool. The precipitate absorbs the urinary pigments, urochrome (yellow), and uroerythrin (red). Microscopically, the sediment is finely granular, the granules tending to collect in masses. On heating the specimen over the flame the urates go into solution, but are again precipitated, as the preparation cools. The addition of hydrochloric acid dissolves the deposit; subsequently crystals of uric acid form. The latter are usually colorless. The rapidity with which the uric acid crystals appear varies greatly; often within ten or fifteen minutes they are numerous. Acetic acid also brings the urates into solution, but the formation of uric acid crystals may be somewhat delayed. The quadriurates give a positive murexid test (see p. 10).

2. **Uric Acid.**—Uric acid may separate from an acid urine from the breaking up of the quadriurates into uric acid and biurates. Crystals of uric acid (Fig. 12), usually colored reddish or yellowish-brown, are then deposited, giving rise to the so-called "brick dust" sediment. They may assume a great variety of form when viewed under the microscope. That most frequently encountered is the "whetstone" crystal. It is seen singly or in clusters, often arranged as a rosette The "church-window" shape is not uncommon. Rhombic plates and six-sided prisms are also characteristic. Hexagonal plates are of less frequent occurrence, and may be colorless; morphologically they are indistinguishable from crystals of cystin. The latter, however, do not give the murexid test. Needles of uric acid arranged in sheaves are rare as a spontaneous sediment,

though not infrequently seen after the addition of hydrochloric acid to the quadriurates.

Uric acid crystals are insoluble in acetic and hydrochloric acids. They are unaffected by heating the specimen. The crystals are soluble in sodium or potassium hydrate, and may be reprecipitated by the addi-

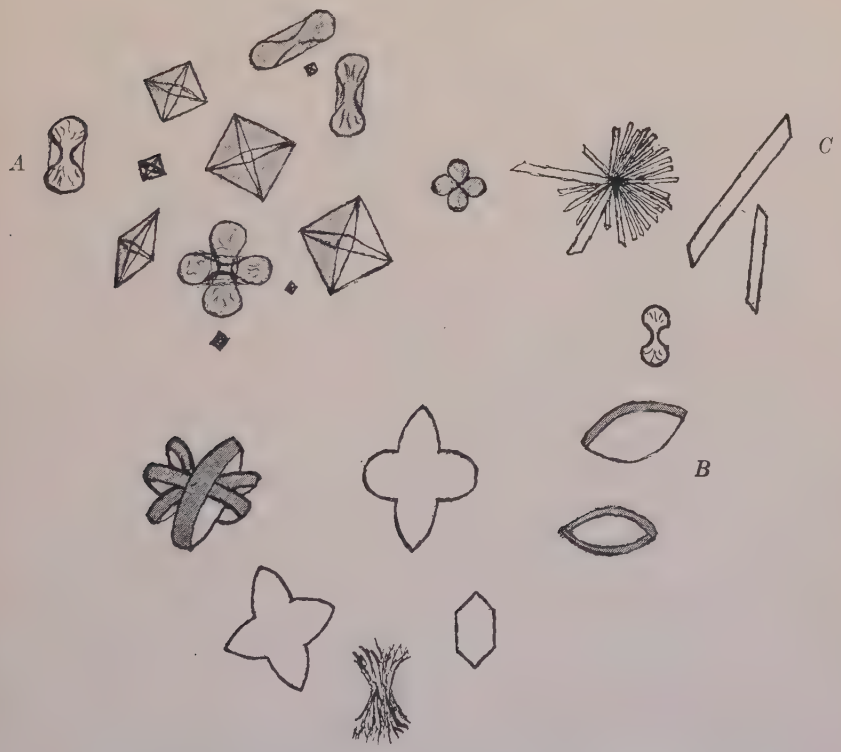

FIG. 12.—URINARY SEDIMENT. *A*, calcium oxalate crystals; *B*, uric acid crystals; *C*, calcium sulphate crystals.

tion of an excess of hydrochloric acid. They give the murexid test (see p. 10).

3. **Calcium Oxalate.**—Calcium oxalate crystallizes most frequently in acid urine, but the crystals remain after the reaction has become alkaline. The crystals (Fig. 12) vary considerably in size; it is often necessary to employ high magnification to recognize them. Most often they occur as small, highly refractive octahedra. Depending upon the position of

the octahedron, its form resembles a square envelope or a lozenge, with lines connecting the opposite angles. Dumb-bell or hour-glass forms, at times with radial striations, and spheroidal masses constitute rarer shapes of calcium oxalate. The crystals are usually colorless, but in icteric urine they may be stained yellow. Calcium oxalate crystals dissolve in hydrochloric or other mineral acid, but are insoluble in acetic acid. The envelope forms may be mistaken for triple phosphate; the latter, however, are readily soluble in acetic acid. Calcium sulphate, calcium carbonate, and uric acid may assume the hour-glass form. Microchemical tests serve to differentiate them from calcium oxalate. (1) Calcium sulphate is insoluble in hydrochloric acid. (2) Calcium carbonate dissolves in acetic acid with the evolution of bubbles of carbon dioxid, which may be seen under the cover glass. (3) Uric acid is insoluble in hydrochloric acid and gives the murexid test.

4. **Calcium Sulphate.**—Calcium sulphate (gypsum) is a rare deposit in very acid urine. It occurs in the form of long, thin, colorless needles, as long, colorless prisms (Fig. 12), often arranged in clusters, or as dumb-bells or hour-glass crystals. Calcium sulphate is insoluble in mineral acids and in ammonia.

5. **Monocalcium Phosphate.**—Monocalcium phosphate, acid calcium phosphate, slender, colorless, rhombic tablets, usually in clusters, resembling somewhat calcium sulphate, is, like the latter, of rare occurrence, and is found in very acid urine. The two are easily distinguished by the solubility of the phosphate in acetic acid and in mineral acids.

6. **Hippuric Acid.**—Hippuric acid crystals are also very rare in the urinary sediment. They are seen as colorless, transparent, four-sided prisms, or as needles (Fig. 13). They are insoluble in hydrochloric acid, which distinguishes them from triple phosphate. From uric acid, which they may resemble somewhat, the crystals are differentiated by the fact that they do not give the murexid test, and that they are soluble in alcohol and ether.

7. **Cholesterin.**—Cholesterin crystals (Fig. 13) may be found in the urine occasionally. They present a characteristic shape, being rhombic plates, often superimposed, with the acute angle notched, as a rule. On the addition of strong sulphuric acid (concentrated sulphuric acid, 5 parts, water, 1 part), the crystals are stained carmine, which later changes to violet. On adding the sulphuric acid with a little Lugol's solution the play of colors is violet, blue, green, and red. The crystals are soluble in ether.

8. Xanthin.—Xanthin crystals have been observed in human urine in only a few cases. They are colorless and, from their shape, may be mistaken for uric acid. They differ from the latter in that they are soluble on heating. They also dissolve in ammonia and give the xanthin test.

Weidel's Test.—On the water bath the crystals are evaporated to dryness in a porcelain dish, to which chlorin water and a trace of nitric acid have been added. The residue, when exposed to ammonia fumes, is stained reddish or purplish-violet.

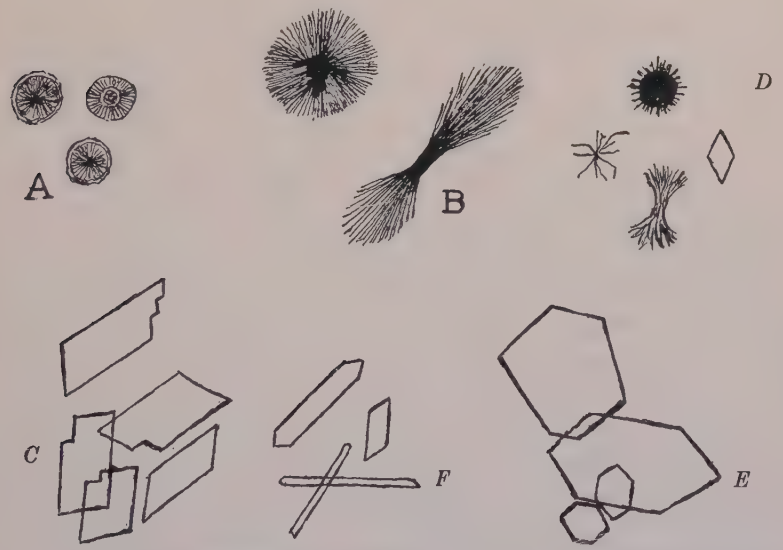

FIG. 13.—URINARY SEDIMENT. *A*, leucin; *B*, tyrosin; *C*, cholesterin crystals; *D*, hematoidin crystals; *E*, cystin crystals; *F*, hippuric acid.

9. Hematoidin.—Hematoidin (*bilirubin*) crystals (Fig. 13) may be found in icteric urine, particularly when it is very acid. They are differentiated from uric acid by the reactions given on page 65. With the murexid test they give a negative reaction. Hematoidin occurs in amorphous masses or as needles, often gathered together to form sheaves, or as rhombs, colored yellow or yellowish-brown.

10. Tyrosin.—Tyrosin (Fig. 13) has been found in very few instances as a spontaneous urinary sediment. It is found precipitated in the form of needles gathered together in bundles like sheaves of wheat. In impure state tyrosin may resemble somewhat spherules of leucin. The crystals

are soluble in alkali, ammonia, and in mineral acids, very slightly soluble in acetic acid. From ammoniacal solution tyrosin crystallizes spontaneously on evaporation.

To obtain tyrosin from the urine[87] the twenty-four-hour specimen is treated with neutral, then with basic, lead acetate, as long as a precipitate forms. The excess of lead in the filtrate is removed by precipitation with hydrogen sulphid. The filtrate is now concentrated to small volume on a water bath. By fractional crystallization tyrosin and leucin (which usually coexist) are separated, since it is chiefly the tyrosin which forms the crystalline deposit. The crystals are then subjected to the tests for tyrosin given below. The leucin which is in the filtrate is converted into its copper salt by boiling with freshly precipitated copper hydroxid. From the hot solution it crystallizes in the form of rhombic plates. The crystals are slightly soluble in water, insoluble in methyl alcohol.

a. Piria's Test.—Dissolve the crystals of tyrosin in warm, concentrated sulphuric acid, permit the solution to cool, then dilute with water, and, finally, neutralize with barium carbonate. The mixture is then filtered, and to the filtrate ferric chlorid solution is added. A violet color appears. The test may fail if free mineral acid remains or if an excess of ferric chlorid be added.

b. Mörner's Modification of Denigès' Test.—To a few c.c. of a reagent (consisting of 1 volume of formalin, 45 volumes of water, and 55 volumes of concentrated sulphuric acid) add the tyrosin crystals or solution and boil. A beautiful green color develops.

c. Hofmann's Test.—A few crystals of tyrosin are placed in a test tube partly filled with water, to which a few drops of Millon's reagent (one part of mercury dissolved in two parts by weight of nitric acid, sp. gr. 1.42, then warm gently, add two volumes of water, and; after standing several hours, obtain the clear supernatant fluid) have been added. On boiling the fluid is stained a beautiful red, and a red precipitate forms.

11. **Leucin.**—Leucin is not seen as a spontaneous sediment in the urine. It is usually present with tyrosin, and may separate as globules resembling fat if the urine be concentrated on a water bath. The globules, unlike those of neutral fat, are insoluble in ether. They are

[87] This and the following tests for tyrosin are taken from Hoppe-Seyler's *Handbuch der chemischen analyse* (H. Thierfelder), Berlin, 1909, pp. 625 *et seq.*, 8th edition.

usually stained brown and present radial striations or concentric rings (Fig. 13), or are hyalin.

To obtain leucin from solution in the urine, see page 86. For its recognition the reader is referred to works on biological chemistry.

12. **Cystin.**—Cystin, a sulphur-containing amino-acid, occurs in the urine in the form of colorless, hexagonal plates (Fig. 13). The presence of the crystals constitutes cystinuria, the manifestation of a rare disturbance of intermediary protein metabolism. Smillie [88] has shown that, if the urine is kept alkaline by cutting down the protein of the food to a low level and giving sufficient sodium bicarbonate to keep the reaction alkaline, the quantity of cystin excreted is unchanged, but the cystin is held in solution. Briefly stated, then, cystin is soluble in alkaline urine, insoluble in acid.

Oftentimes the crystals are superimposed or overlap one another. Uric acid at times assumes the identical crystalline form and may be colorless. The two may be distinguished by the fact that cystin is readily soluble in hydrochloric acid and ammonia; further, by the fact that the murexid test is not given by cystin. Cystin crystals are insoluble in acetic acid, alcohol, and ether. When the crystals are atypical they may be reprecipitated from ammoniacal solution by the addition of acetic acid. Microscopic examination should then reveal characteristic crystals.

To isolate cystin in solution the urine is treated with neutral, then with basic, lead acetate (see under tyrosin, p. 86). The filtrate is concentrated on a water bath. Cystin separates on prolonged standing or after the addition of an excess of acetic acid.

Qualitative Test.—Boil a portion of the urine with sodium or potassium hydrate and lead acetate. A black color arises from the sulphid of lead which is formed. Albumin or other proteins, if present, must first be removed.

Sediments in Neutral or Alkaline Urine

In addition to the crystals described in acid urine, which frequently persist after the reaction has become alkaline, there are a number which are commonly found in alkaline urine.

1. **Tricalcium and Trimagnesium Phosphates.**—Tricalcium and trimagnesium phosphate, the *amorphous* phosphates, are recognized as white

[88] Smillie, W. G. "Treatment of Cystinuria." *Arch. Int. Med.*, 1915, XVI, 503.

or grayish-white deposits, often very abundant, which are easily soluble in hydrochloric and acetic acids. The lack of coloration and the fact that they do not dissolve on heating the preparation differentiate them from the quadriurates, which they resemble somewhat microscopically. The murexid test is negative, a further differential point. Furthermore, they are soluble in acids.

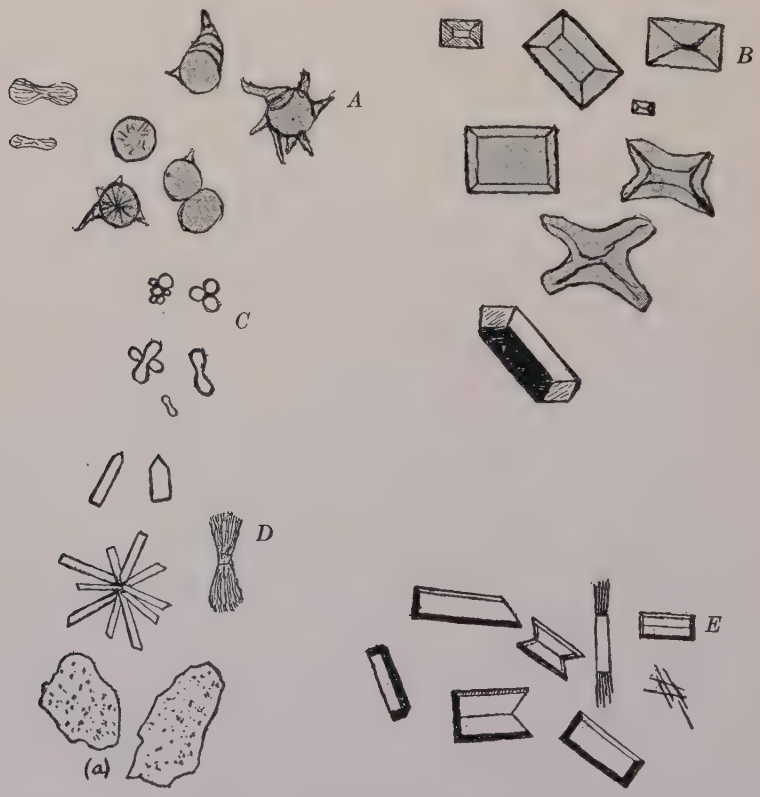

FIG. 14.—URINARY SEDIMENT. *A*, crystals of ammonium biurate; *B*, crystals of ammonium magnesium phosphate; *C*, calcium carbonate; *D*, neutral (dicalcium) phosphate; *E*, neutral (dimagnesium) phosphate.

2. **Calcium Carbonate.**—Calcium carbonate is also usually amorphous, and is generally found mixed with the amorphous phosphates. It differs from the phosphates in the fact that the addition of acid causes solution with the evolution of carbon dioxid. The salt may also appear as dumb-bells or spheres with radiating lines (Fig. 14), resembling similar

forms of calcium oxalate, calcium sulphate, and uric acid. Its solubility in acids with gas formation identifies it as calcium carbonate.

3. **Ammonio-magnesium Phosphate.**—Ammonio-magnesium phosphate, "triple" phosphate, is the crystal most commonly observed in alkaline urine. For its formation it is necessary that ammonia be produced. It therefore happens that the crystals are occasionally encountered while the reaction of the urine is still acid, though their number rapidly increases with the progress of ammoniacal fermentation. The crystals (Fig. 14) belong to the rhombic system. The "coffin-lid" is the commonest form. Erosion of these produces the irregular X-shaped crystals. With good illumination triple phosphate crystals have a greenish tint. They vary greatly in size; at times they are so large that they are visible with the unaided eye. Some of the smallest crystals, when perfect, resemble somewhat the envelope forms of calcium oxalate. Their solubility in acetic acid is a differential point. When the phosphates are precipitated artificially with ammonia, fern-like crystals are usually found. In a native sediment, particularly when it has stood for some time, it is customary to find the majority of the crystals imperfect.

4. **Ammonium Biurate.**—Ammonium biurate, like triple phosphate, is deposited only as a result of the liberation of ammonia in the urine. It forms balls or spheres of yellow or light brownish color, often with striations, oftener with horny projections or processes, producing the so-called "thorn-apple" or "morning star" crystals (Fig. 14). Their shape may be anything, depending on the number, position, and length of the projections. The crystals are soluble in acetic and hydrochloric acids, with the subsequent formation of uric acid crystals. They give the murexid test.

It is not uncommon to find amorphous phosphates and carbonates, triple phosphate, and ammonium biurate combined in the sediment of an ammoniacal urine.

5. **Neutral Magnesium Phosphate.**—Neutral magnesium phosphate, dimagnesium phosphate (Fig. 14), is a very rare sediment, which is met with in weakly alkaline urine. It forms long, refractive, rhombic plates. On treating it with 20 per cent ammonium carbonate solution the crystals become opaque and the edges eroded. They are easily dissolved in acetic acid, and may be reprecipitated by the addition of sodium carbonate.

6. **Neutral Calcium Phosphate.**—Neutral calcium phosphate, dicalcium phosphate (Fig. 14), is very infrequently met with in weakly acid,

neutral, or weakly alkaline urine. It gives rise to colorless wedges or prisms, usually clumped together. The crystals are soluble in acetic acid. On treating them with 20 per cent ammonium carbonate, balls of calcium carbonate are produced.

THE MICROCHEMICAL REACTIONS OF SEDIMENTS TO REAGENTS

The reactions described above may be summarized as follows:

1. *Strong acetic acid* dissolves calcium and magnesium phosphates, ammonio-magnesium phosphate, and calcium carbonate, the last with the evolution of gas. It does not dissolve calcium sulphate, calcium oxalate, uric acid, cystin, tyrosin (very slightly soluble), and xanthin. Salts of uric acid are slowly eroded, and after several hours crystals of uric acid are deposited.

2. *Hydrochloric acid* dissolves all crystals excepting uric acid, hippuric acid, and calcium sulphate.

3. *Ammonium hydrate* dissolves cystin, tyrosin, and xanthin. Uric acid crystals are partially eroded with the formation of ammonium biurate. Calcium phosphate, calcium sulphate, and calcium oxalate, and the salts of uric acid are unaffected by ammonia.

4. *Water* in large amount dissolves calcium sulphate; but many other crystals are not wholly insoluble in water—uric acid and its salts, triple phosphate, tyrosin, and xanthin.

5. *Alcohol* dissolves tyrosin, leucin, cystin, and hippuric acid.

6. *Chloroform* dissolves bilirubin (hematoidin) and fat.

The Organized Sediments

1. **Epithelial Cells.**—Epithelial cells (Fig. 15) are normally found in the urine, due to the fact that the cells of the genito-urinary mucosæ are constantly desquamating. As a rule, the cells are few in number and the majority of them may be caught in the mucous threads of the nubecula, if the specimen be allowed to stand a short time. In the case of women, however, the urine frequently contains a macroscopic sediment composed largely of enormous numbers of epithelial cells, derived chiefly from the vagina.

A variety of form may be noted in the epithelial cells of the urine. The vaginal cells are rather large, squamous cells with relatively small, round or oval nuclei. Sheets of these cells are often shed *en masse*.

CRYSTALS IN URINE *

Reaction	Crystals	Shape	Color	Solubility				
				Heat. sol	Acetic	HCl	NH_4OH	KOH
Acid	Amorphous Urates		Yellow to Rose Red	+	+ Uric Acid	+ Uric Acid	−	−
	Uric Acid		Colorless Yellow to Brown	−	−	−	+	+
	Calcium Oxalate	Dumb bells Envelopes	Colorless	−	−	+	−	−
	Calcium Sulphate	Needles	Colorless	−	−	−	−	−
Neutral	Ammonium Biurate	Morning Star	Yellow Brown	− −	+ Uric Acid	+	−	−
	Di-calcium Phosphate	Needles Wedges	Colorless	−	+	+	−	−
Alkaline	Ammonium Biurate	Thorn-apple Morning Star	Yellow Brown	−	+	+	−	−
	Amorphous Phosphates		Light	−	+	+	−	−
	Amorphous Carbonate		Light	−	+ CO_2	+ CO_2	−	−
	Calcium Carbonate	Dumb bell	Light	−	+ CO_2	+ CO_2	−	−
	Triple Phosphates	Coffin lid	Light	−	+	+	−	−
	Neutral Magnes. Phosph.		Light	−	+	+	−	−

* This table has been prepared by Dr. James S. Brotherhood, to whom the author is indebted for permission to publish it.

Cells derived from the kidney are usually round or cuboidal, with large, vesicular nucleus.

The protoplasm of the epithelial cells is prone to undergo *fatty degeneration*. The microscopic appearance is fairly characteristic. The droplets differ in size and may be few or numerous; at times the cell is completely filled, and it may be impossible to demonstrate the nucleus. The fat droplets are stained a deep orange with Sudan III or Scharlach R.

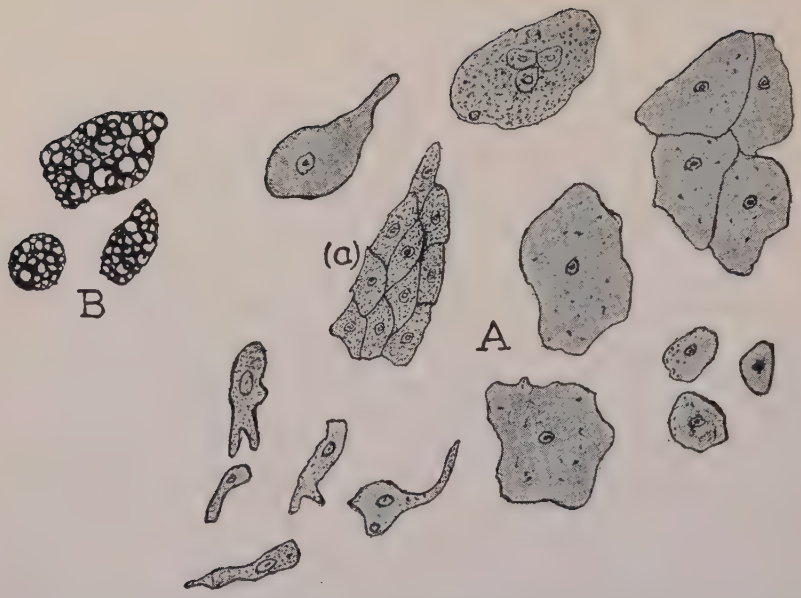

FIG. 15A.—URINARY SEDIMENT. *A*, epithelial cells seen in the urinary sediment (*a*) vaginal epithelia; *B*, epithelial cells containing fat droplets.

FIG. 15B.—URINARY SEDIMENT. *A*, red blood cells; *B*, crenated red cells; *C*, laked red cells "shadows;" *D*, eosinophile cell; *E*, *F*, pus cells; *G*, cell containing fat droplets.

Occasionally *myelin* or albuminous granules are present in the protoplasm.

Since it is quite generally agreed that it is impossible, from their morphology, to determine the origin of epithelial cells seen in the urine, detailed description of them is superfluous.

Epithelial cells are distinguished from *pus cells* by the shape of the nucleus. The epithelial cell possesses a single round or oval nucleus; rarely, in disease, isolated multinucleated cells are observed. The pus cell, on the other hand, has a polymorphous nucleus. In the fresh sediment the nuclei are not easily seen; they stand out sharply after the addition of dilute (3 per cent) acetic acid. Staining is somewhat less satisfactory.

"Heart-failure" cells have recently been described in the urine.[89] Like those seen in the sputum, they are epithelial cells, which are laden with altered blood pigment. The pigment granules are light golden yellow in color. At times there is a diffuse yellowish staining of the cells in the absence of icterus. The cells are not uncommon with chronic passive congestion of the kidneys, but they are not diagnostic of the condition; they *may* be found in hematurias unassociated with passive congestion.[90] The cells are usually more or less swollen, of varying size, often very large, and at times a large, round nucleus is visible (Bittorf). They are frequently much degenerated.

2. **Pus.**—In health the urine may contain isolated pus cells (Fig. 15), though they are ordinarily missed altogether. An exception is not infrequently met with in women with leukorrheal discharge. The vaginal secretions become mixed with the urine and, as numerous pus cells may be present in the former, they are, of course, found in the examination of the urinary sediment. It is, therefore, necessary in such cases to thoroughly cleanse the external genitals before collecting the specimen or to obtain the urine by means of a catheter. The second procedure is the more accurate method and is to be preferred. The presence in the urine of abnormal numbers of pus cells (Fig. 15) gives rise to the condition designated *pyuria*. When only a few cells are present, there is no macroscopic alteration in the appearance of the urine, but marked pyuria causes a turbidity, and in extreme cases the urine may even appear creamy.

[89] Bittorf, A. "Ueber Herzfehlerzellen im Harn." *München. med. Wchnschr.*, 1909, LIX, 1775.

[90] Koller, E. "Zum Vorkommen von 'Herzfehlerzellen' im Harn." *Wiener klin. Wchnschr.*, 1911, XXIV, 636.

Pyuria has been observed in:

1. *Acute inflammations* in any part of the genito-urinary tract; thus, in acute urethritis, cystitis, pyelitis, nephritis, from whatever cause.
2. Many *chronic inflammations* of the genito-urinary tract.
3. *Abscess* rupturing into the urinary passages.
4. *Leukorrhea* (excluded by catheterization).

The pus cells (polynuclear neutrophilic leukocytes) retain their characteristic morphology well in acid urine. When the urine becomes strongly alkaline, the pus forms a ropy, tenacious mass, in which the individual cells are swollen, often distorted, and so greatly degenerated that they may be no longer recognizable. However, in weakly alkaline, amphoteric, or weakly acid urines the cells are generally very well preserved, and may even exhibit ameboid activity.

Microscopically, the protoplasm of the cells is finely granular. The majority of the granules are the neutrophilic granules of the cell, though fat droplets may be more or less abundant. In unaltered cells the diameter is about 12 micra—rather smaller than most of the epithelial cells. To determine the nature of the cells beyond question it is necessary to demonstrate the typical nuclei. This is best accomplished by the addition of 3 per cent acetic acid; the polymorphous nuclei are then sharply defined. The specimen is examined with the high-power dry objective. Staining the sediment may be tried, but is less satisfactory. Carbol-thionin is one of the best stains for this purpose.

In following a patient with pyuria, it may be desirable to *count the pus cells* in the urine from time to time. For this purpose the twenty-four-hour specimen should be used, and care must be exercised to prevent bacterial ammoniacal fermentation, otherwise the cells become glued together, making a count impossible. The best chemical preservative for this purpose is formalin. Commercial 40 per cent formalin is added in sufficient quantity to give a solution of 1 to 2 per cent, the preservative being added to each portion of urine as it is collected. Such a procedure is possible only in a hospital, as a rule; when it cannot be carried out, 15 to 20 c.c. of formalin may be placed in the bottle in which the urine is collected. The formalin prevents bacterial growth and at the same time renders the cell nuclei more prominent. Its disadvantage lies in the fact that the cells are clumped together in certain instances. If an ice chest is available, simple refrigeration is to be preferred to any other

method of preservation. The urine, if neutral or alkaline, is acidified with acetic acid. The specimen is well stirred to secure a uniform suspension of the cells, and the count is then made directly from it with the hemocytometer, employing the technic used for counting the blood. If the cells are very numerous, it will be found more convenient to use the red pipette.

With the escape of a purulent exudate into the genito-urinary tract, the albumin of the exudate becomes mixed with the urine, constituting a *false albuminuria,* if the lesion is extrarenal. It is often difficult to interpret findings when a false albuminuria, such as this, is met with. The question arises whether the albumin is derived entirely from the purulent exudate or in part from the kidneys (true renal albuminuria). The presence or absence of casts is of value in determining the latter; instrumental examination may be decisive. Posner [91] has recorded observations which show that, with 80,000 to 100,000 pus cells per cubic millimeter of urine, only about 0.1 per cent albumin is added to the urine. By comparing the cell count with the quantity of albumin, the source of the latter may be determined approximately.

The following chemical tests for pus may be applied to the urine or to the sediment:

a. The Guaiac Test.—Equal parts of hydrogen peroxid and freshly prepared tincture of guaiac (see p. 72), when layered over the urine, cause a blue ring to appear at the line of contact in the presence of pus. It may be necessary to wait a few minutes for the color to appear. The color disappears on boiling, unlike that caused by the presence of blood. The test is quite delicate, but is not specific.

b. Meyer's Guaiac Test [92] (adapted to the urine).—A drop or two prepared tincture of guaiac (see p. 72), when layered over the urine, thirds full of water. The contents of the tube are well mixed and allowed to extract a few minutes, in order to liberate the oxidizing enzyme of the pus cells. The fluid is halved. On one portion freshly prepared tincture of guaiac (*without* hydrogen peroxid) is superimposed carefully, and at the line of contact a blue ring appears, which fades in the course of about a half hour. The remaining portion is boiled actively for two

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[91] Posner, C. "Ueber Harntrübung." *Deutsche med. Wchnschr.,* 1897, XXIII, 635.

[92] Meyer, E. (a) "Beiträge zur Leukocytenfrage." *München. med. Wchnschr.,* 1903, L, 1489. (b) "Ueber die cytodiagnostische Bedeutung der Guajakreaktion." *Ibid.,* 1904, LI, 1578.

to three minutes, then cooled and treated with tincture of guaiac in the manner just described. Boiling destroys the ferment, and the test is therefore negative. The test is delicate, and points definitely to the presence of an oxidase; in the urine the only common source of oxidase is pus. If much albumin is present, the reaction may be inhibited.[93]

3. **Eosinophiles.**—Eosinophilic leukocytes are occasionally found in the urine (Fig. 15), especially when there is a blood eosinophilia.

4. **Blood.**—Red blood corpuscles are never found in normal, voided urine, excepting the admixtures of blood which occur during menstruation. *Hematuria* is the term used to signify blood in the urine. A small number of blood corpuscles produce no visible change in the appearance of the urine. With larger quantities the translucency of the urine is lost, it becomes "smoky" in appearance on agitating the specimen, and darker in color. A reddish-brown sediment composed largely of red cells may settle out.

Hematuria results when there is a hemorrhage into any part of the genito-urinary tract.

1. *Renal.*—The blood is thoroughly mixed with the urine, and blood casts are usually seen microscopically.
   - *a.* In *acute nephritis* and during *acute exacerbations* of chronic nephritis.
   - *b.* In the *hemorrhagic or malignant forms* of the *acute infectious diseases,* as small-pox, measles, scarlet-fever, typhoid fever, malaria.
   - *c.* In *hemorrhagic diseases,* such as acute leukemias, hemophilia, scurvy, and purpuras.
   - *d.* In *renal infarction.*
   - *e.* From *irritation* by substances introduced into the body, such as *bichloride of mercury, turpentine,* etc.
   - *f.* With *new-growths* (carcinoma, sarcoma).
   - *g.* In *tuberculosis* of the kidney.
   - *h.* In *echinococcus* disease of the kidney.
   - *i.* In *cystic kidney,* at times.
   - *j.* In *renal calculus.* In this condition, the blood may clot, forming a cast of the pelvis of the kidney.

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[93] Watson, Helen. Personal communication.

k. In *renal epistaxis,* usually unilateral [94] (*"essential hematuria"*).
l. From *trauma.*
m. In *pyelitis* at times.
n. In the *"erythema group" of diseases,* at times.

2. *Ureteral.*—With a large hemorrhage, blood casts of the lumen of the ureter may be passed.
   a. Calculi.
   b. Neoplasms.
   c. Tuberculosis or *other infections.*
   d. Trauma.
3. *Vesical.*—The site of the hemorrhage is best determined by cystoscopic examination; in *renal* and *ureteral* hemorrhage, *ureteral catheterization* is of the greatest aid in diagnosis. With vesical bleeding, in the two-glass test, the portion passed into the second glass contains more blood.
   a. Neoplasms.
   b. Acute inflammations.
   c. Varicosities.
   d. Calculi.
   e. Parasitic, especially frequent in infections with *Schistosoma hematobium.*
   f. Trauma.
   g. After certain *irritants,* such as hexamethylene.
4. *Urethral.*
   a. Acute inflammations.
   b. Trauma.
   c. Neoplasms (rare).

In women, menstrual flow must be excluded, by catheterization, if necessary.

The chemical tests for blood are given in connection with hemoglobinuria (p. 70 *et seq.*).

The microscopic examination is made with the high-power dry objective. The erythrocytes (Fig. 15) may be well preserved, and exhibit their characteristic morphology and color. If laking of the cells has occurred, the majority, or all, of the cells appear as "shadows," i.e., the

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[94] Quinby, W. C. "The pathology of the renal pelvis in two cases showing hematuria of the so-called essential type." *Jour. Urol.,* 1920, IV, 209.

coloring matter has escaped from the red cell, and only the cell membrane remains. To detect the shadows it is essential that the light be cut off as much as possible. In concentrated urine crenation of the red cells, giving rise to thorn-apple forms, is observed.

5. **Casts.**—The occurrence of casts (Fig. 16) in the urine, cylindruria,[95] is very frequent in disease, and may also be observed in old age and in association with so-called physiological albuminurias. The casts are derived from the renal tubules. They vary greatly in size, the longest measuring in the neighborhood of 1 mm. The thickness of casts is also variable, but in a given cast the width is quite uniform.

Casts are found in the urine (cylindruria), as a rule, whenever there is an albuminuria of *renal* origin (see p. 25). Occasionally, in the physiological albuminurias, no casts are discoverable. On the other hand, a few hyaline casts may be encountered in the absence of albuminuria, especially in cases of chronic interstitial nephritis.

It is important to remember that all varieties of casts, even blood and pus casts, may be found in the urine after severe physical exertion, such as a Marathon race, for example. With normal kidneys, the reaction subsides within a day or so.

a. Epithelial casts (Fig. 16, *A* see also *D*), are composed of renal epithelial cells in whole or in part. Any cast to which one or more renal epithelial cells are attached may conveniently be designated epithelial (Emerson). The cells are not of equal size, some being large, others smaller. They have a round or oval nucleus, and are usually flat and polygonal. In most instances the protoplasm of the cells is degenerated, showing fat droplets, albumin granules, or, more rarely, myelin droplets. It is unusual to find true epithelial cylinders possessing a distinct lumen. To distinguish between epithelial and pus cells it is necessary to demonstrate the morphology of the cell nuclei by the addition of dilute acetic acid. The cast may be *mixed,* i.e., it may contain both epithelial and pus cells, it may be partly cellular, partly granular, etc.

b. Pus casts (Fig. 16, *B,* see also *D*) like epithelial casts, consist in whole or in part of pus cells. The cells are generally smaller and rounder than the epithelial cells. Their protoplasm is finely granular, but is subject to the same degenerations as that of epithelial cells. The cells are characterized by their polymorphous nuclei, which are usually

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[95] Emerson, C. P. "Cylindruria." *Jour. A. M. A.*, 1906, XLVI, 5; 89.

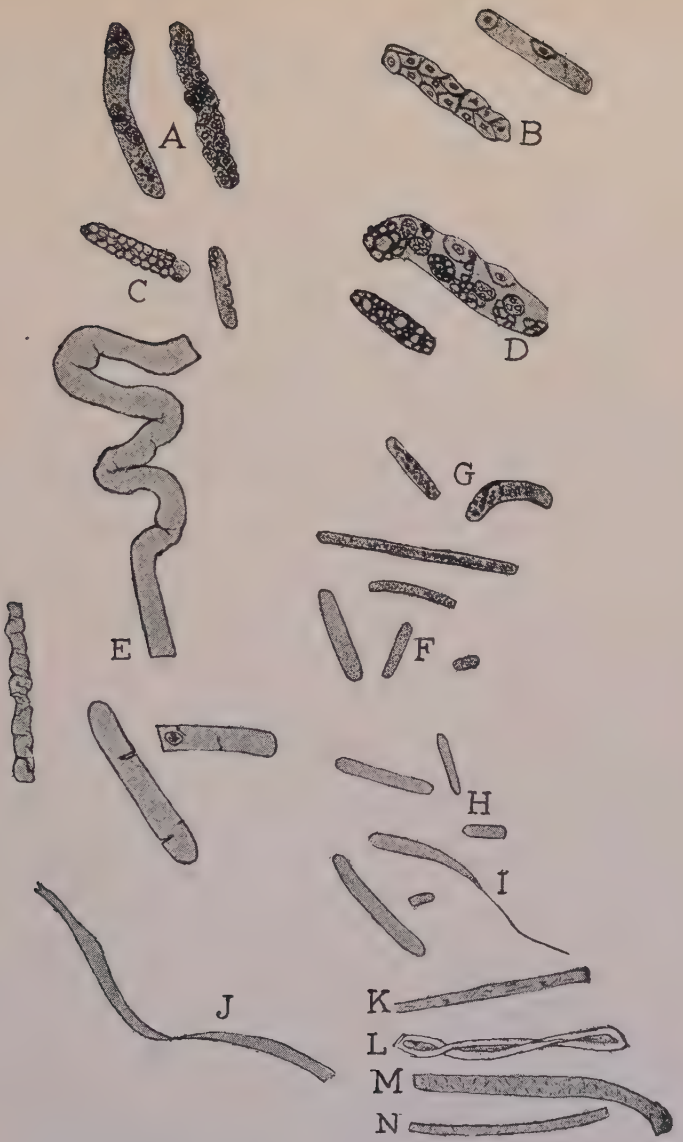

FIG. 16.—URINE SEDIMENT. *Casts*. *A*, pus casts; *B*, epithelial casts; *C*, blood casts; *D*, fatty casts, the larger also a mixed cell cast, with pus and epithelial cells; *E*, waxy casts, one with a pus cell attached; *F*, finely granular casts; *G*, coarsely granular casts; *H*, hyalin casts; *I*, cylindroid; *J*, mucous thread; *K*, artefact: scratch on glass slide; *L*, contamination, cotton thread; *M*, contamination, wool thread; *N*, contamination, linen thread.

visible only after treating the specimen with dilute acetic acid or a dye. At times the cells are so degenerated that the nuclei are no longer demonstrable.

c. Blood casts (Fig. 16, *C*) when pure, are clots which form in the renal tubules. However, any cast in which one or more blood cells are visible is designated a blood cast. At times the red blood corpuscles are not well preserved; shadows of red cells and granules or crystals of hematoidin may be attached to the cast.

d. Fatty casts (Fig. 16, *D*) result from the fatty degeneration of the cells of epithelial casts, or, less commonly, of pus casts. Often the outlines of the original cells are preserved. The casts usually have a yellowish or even brownish tint. The droplets vary considerably in size, some being almost as large as a cell. Ether dissolves the fat droplets, and they may be stained by adding Sudan III or Scharlach R to the preparation. Occasionally fatty acid needles project from the cast.

e. Coarsely granular casts (Fig. 16, *G*) are whitish, yellow, or very dark brown in color, quite opaque, and are covered, either partly or entirely, by coarse granules, as their name indicates. Some of the granules dissolve in ether and stain with osmic acid or other fat stains, while others are albuminous and are soluble in acetic acid. Occasionally granules resembling myelin droplets are observed. Coarsely granular casts are probably derived from epithelial and pus cell casts. All stages in transition may be seen. The casts are often partly waxy.

f. Finely granular casts (Fig. 16, *F*) resemble the coarsely granular, but they are much less opaque and the granules are much finer. Transitions from the coarsely to the finely granular are met with. The granules may cover part or all of the cast. The non-granular portion of the cast may be cellular; more frequently, it is hyalin. Fat droplets are of much less frequent occurrence than in the coarsely granular variety, and myelin droplets are exceptional. The finely granular casts are best seen with low illumination. They are one of the commonest types of cast in disease.

g. Hemoglobin casts are very rare and are always associated with hemoglobinuria. They are covered with dark, granular pigment; less commonly needles of hematoidin are attached to the casts.

h. Waxy (*colloid or amyloid*) *casts* (Fig. 16, *E*) are opaque, very refractive, and white or yellowish in color. Their appearance suggests bodies made of paraffin or wax, according to the color of the cast. They are very brittle, and not uncommonly present transverse fissures or cracks. During centrifugalization they may be broken; the fragments are then

seen in the sediment. Some waxy casts give the iodin reaction for amyloid when treated with Lugol's solution. It is not unusual to find the cast in the form of a spiral or corkscrew. Cells may be attached to waxy casts, or they may be granular in part. They are supposed to be derived from coarsely granular casts or from hyalin casts which have remained in the renal tubules for some time.

i. Hyalin casts (Fig. 16, *H*) are pale and very slightly refractive. Unless most of the light is cut off, it is impossible to see them; they are almost glassy in their translucency. They may be stained by dilute gentian violet or by Lugol's solution, and are then easily found. They are usually narrow and have rounded ends. In a urine undergoing ammoniacal fermentation they disappear more rapidly than any other kind of cast. Between the glassy hyalin cast and the waxy cast there is a large intermediate group, consisting of casts which are much less opaque than waxy casts, but—though designated hyalin—possessing considerably more density than the glassy type of hyalin cast. Hyalin casts are supposed to represent albumin coagula from the renal tubules or a morbid, coagulable secretion of the renal cells. They are the commonest variety of cast.

Upon any cast granular, amorphous urates may be deposited, producing a finely granular appearance. Bacteria in large number, attached to a cast, produce a somewhat similar picture. The uniform outline of the cast is often lost, and the artefact may be recognized also by microchemical reactions.

j. Cylindroids (Fig. 16, *I*) are casts, one of whose ends tapers to a thread-like filament. They are usually hyalin, though at times granular, and have the same significance as casts.

Under the name *pseudocast* is included anything which may be mistaken for a cast. Scratches on the glass slide most often mislead the beginner. Particles of dust, fibers (Fig. 16, *K, L, M, N*), etc., may also cause confusion. The morphology of the cast is not duplicated, and experience soon teaches one to differentiate.

6. **Mucous Threads.**—Mucous threads (Fig. 16, *J*), sometimes included with cylindroids, with which they have nothing in common, are normally present in the urine. They make up the nubecula. They appear as long, narrow, translucent bands of mucin, of unequal thickness, often twisted or folded like a ribbon, at times branched. Nearly always a few epithelial cells and, perhaps, an occasional pus cell are attached to the threads. Unlike hyalin casts and cylindroids, mucous threads

are insoluble in acetic acid. The length of the threads is at times great; a single specimen may extend through several fields of the microscope.

7. **"Clap Threads."**—Clap threads (*Tripperfäden*) are white or grayish-white, thread-like bodies which are seen floating in the urine, when the specimen is agitated. They are not always gonorrheal in origin, as the name suggests, though in the vast majority of instances associated with a chronic specific urethritis. They are ½ to 1 cm. or more long, and consist of a matrix of mucus, in which epithelial or pus cells or both are embedded. The pus cells may be so abundant that the thread is quite opaque and yellowish. In the gonorrheal cases it is often possible to demostrate the presence of the gonococcus.

MICROÖRGANISMS IN THE URINE

Gonococcus.—The gonococcus (Fig. 17) is a diplococcus shaped like a biscuit or coffee bean. It is found either within the pus cells or free in the serum. It is Gram-negative. Therefore, the finding of a diplococcus in urethral pus, of characteristic morphology, which decolorizes with Gram's stain, makes it highly probable that the organism is the gonococcus.

To demonstrate the gonococcus the following procedure may be employed:

1. A smear of the pus is made on a glass slide. It is dried in the air and fixed by passing it through the flame of a Bunsen burner five or six times.

2. Stain 1 to 3 seconds with anilin water gentian violet. (Avoid overstaining.) [To prepare anilin water gentian violet, 10 parts of anilin oil are thoroughly shaken with 100 parts of water, and, after standing about five minutes, the rather milky emulsion is filtered through a moistened filter paper. The filtrate should contain no large oil droplets. Now add 11 parts of saturated alcoholic solution of gentian violet and 10 parts of absolute alcohol. The solution keeps not longer than eight to ten days (Schmorl)].

3. Wash the preparation immediately in tap water and blot it till dry.

4. Add a drop of immersion oil, and examine the specimen microscopically for diplococci. If none is found after examining three slides carefully, the chances are that diplococci are not the etiological factor in the production of the purulent exudate. If suspicious organisms are

seen, it becomes necessary to determine whether they are Gram-negative, i.e., whether they are decolorized after treating them with Gram's iodin solution. The further steps are:

5. Removal of the oil by wiping the specimen with xylol.

6. The specimen is now covered with Gram's iodin solution, one to two minutes (iodin, 1.0 gm.; potassium iodid, 2.0 gm.; distilled water, 300.0 c.c.), and then, without washing in water, it is transferred to—

7. Absolute alcohol, in which it is decolorized, until the specimen

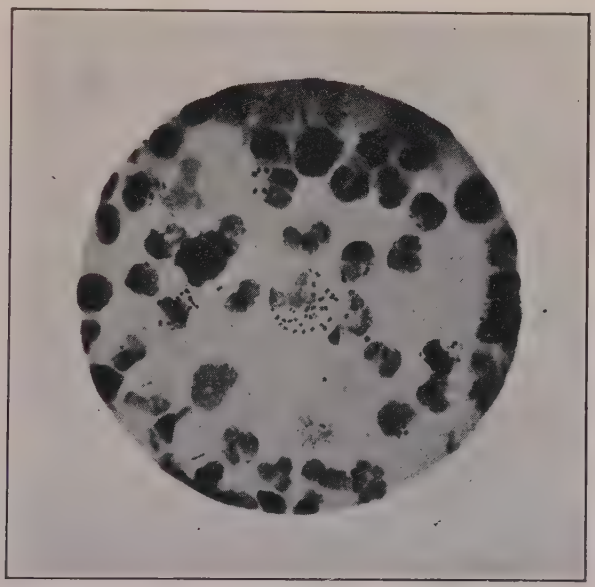

FIG. 17.—GONOCOCCI IN PURULENT EXUDATE; ORGANISMS ALMOST ALL INTRACELLULAR. ×1000 (after Wm. B. Wherry, from photomicrograph by Chas. Goosmann).

is colorless or yellowish-gray, excepting the thickest parts of the smear, which may still retain a little blue. Blot dry.

8. Counterstaining may be performed with Bismarck brown (vesuvin). [The stain is prepared by dissolving 2 gm. of the powdered stain in a mixture composed of 60 c.c. of 96 per cent alcohol and 40 c.c. of distilled water. The solution is boiled carefully, and, after it has cooled, is filtered. To prevent bacterial growth, a few drops of carbolic acid may be added (Schmorl)]. The stain is allowed to act one to two minutes.

9. Wash in water, dry, and examine.

After the first staining all bacteria and cell nuclei are colored purple by the gentian violet. Decolorization removes the dye from all Gram-negative bacteria and from the cell nuclei. The counterstaining with Bismarck brown then stains the Gram-negative bacteria and nuclei brown, whereas the Gram-positive organisms retain the violet.

The diagnosis of gonorrhea is made, therefore, by finding the Gram-negative diplococci in the pus from the *urethra;* the organisms are found intracellularly and extracellularly. The more acute the case, the easier the diagnosis as a rule. The characteristic intracellular arrangement is usual in *acute* cases; in very *chronic* cases the organisms are very scarce and may not be found, making a negative result inconclusive. In such cases, complement fixation may be helpful; cultural examination may be required, the usual medium being one containing two to three parts of meat infusion-agar with one part of uncoagulated human ascitic fluid, hydrocele fluid, or blood serum.[96]

In the examination of pus from the *eye* or from the *vagina,* the finding of Gram-negative cocci is of less diagnostic value, because of the fact that other Gram-negative cocci are found in these parts occasionally. *Micrococcus catarrhalis* and the *meningococcus* may be encountered in the eye. In vaginitis, abscess of Bartholin's gland, etc., one must rule out the anaerobic Gram-negative diplococcus, *Micrococcus reniformis,* which resembles the gonococcus in its morphology.[97]

Treponema pallidum (Synonyms: *Spirochæta pallida, Spironema pallidum, Microspironema pallidum*).—*Treponema pallidum* (Fig. 18), discovered by Schaudinn in 1905, has been shown to be the cause of syphilis. The spirochetes are 4 to 10 micra long [98] and up to 0.5 micron in width (Castellani and Chalmers). The organisms have a spiral form, with six to twelve or more turns in the spiral. In the fresh specimen the spirochete has a screw-like motion, but, despite its motility, its position in the field remains almost stationary. It is distinguished from

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[96] Cultivation of the gonococcus is attended by many difficulties and uncertainties, and cannot be undertaken with much hope of success except by those with special training in bacteriologic technic. An excellent method is that described by Wherry, Wm. B., and Oliver, Wade W. "On a rapid method of cultivating the gonococcus." *Jour. Infect. Dis.,* 1916, XIX, 288.

[97] Oliver, Wade W., and Wherry, Wm. B. "Notes on some bacterial parasites of the human mucous membranes." *Jour. Infect. Dis.,* 1921, XXVIII, 341.

[98] Neveu-Lemaire gives the length as 6 to 14 micra, the thickness 0.25 micron or less.

other spirochetes (*S. refringens*), which may be met with on ulcerated surfaces, by the difficulty with which it is stained and by the number and permanence of the spirals.

The spirochetes are demonstrable in the primary and secondary lesions of syphilis. The surface of the lesion is first cleansed to remove *S. refringens* and other contaminations as much as possible, and then, if necessary, the lesion is slightly scarified or rubbed with sterile gauze. By pressure or, better still, by suction apparatus, a drop of serum, usually slightly blood-tinged, is obtained. Many red corpuscles render the examination difficult, and are to be avoided. The serum may be examined

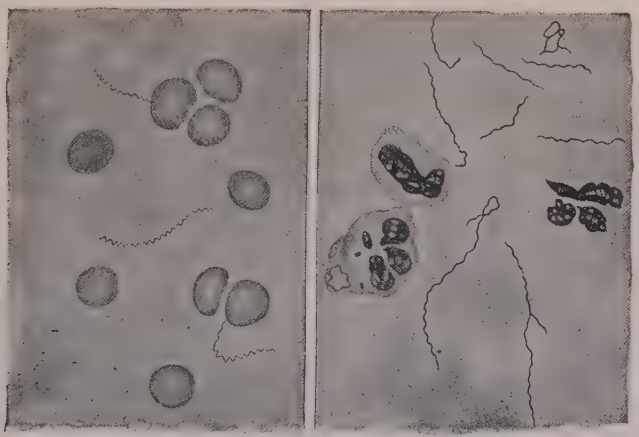

FIG. 18.—TREPONEMA PALLIDUM (Spirochæta pallida) on the left; SPIROCHÆTA REFRINGENS on the right (after Emerson)

in the fresh state with a dark field illuminator, or preparations may be stained. The organism stains faintly and is difficult to see, as its specific name indicates.

STAINING METHODS.—Smears of the serum are prepared on glass slides or cover glasses, and allowed to dry in the air.

1. The smears may be fixed and stained with many of the modifications of the Romanowsky stain, such as Wright's, Leishman's, Wilson's, Hasting's.[99] The technic is the same as that used in staining the blood (p. 285). The *Treponema pallidum* is usually stained a faint blue,

[99] Geraghty, J. T. "The practical value of the demonstration of Spirochæta pallida in the early diagnosis of syphilis." *Bull. Johns Hopkins Hosp.*, 1908, XIX, 364.

but occasionally has a pinkish color, while *Spirochæta refringens* is stained a deep blue.

2. *Giemsa's stain* is also a modification of the Romanowsky stain. It has been employed extensively in searching for the spirochetes. Of the numerous methods of using it, the following are recommended:

a. The specimens [100] are fixed by immersion in absolute alcohol 15 to 20 minutes or by passing through the flame three times. Ten drops of Giemsa's stain (Grübler's mixture) are then mixed with 10 c.c. of distilled water, shaking after the addition of each drop of stain. (The dilution must be freshly prepared each time the stain is used.) Cover the specimen with the diluted stain, warm it till a slight steam arises, allow it to cool about 15 seconds; the stain is then poured off, and replaced by more of the diluted stain. This procedure is repeated four or five times, when the specimen is washed, dried, and mounted in balsam. The parasites are stained dark red. The slide must be free from grease, and the receptacle for the diluted stain and the staining forceps must be free from acid or precipitated stain. The water used for washing must not be acid.

b. Giemsa's [101] azure-eosin staining mixture (Grübler's make) is diluted with an equal volume of pure methyl alcohol (Kahlbaum's or Merck's) and placed in a dropping bottle. It is well to prepare only a small quantity at a time, as it is not known how permanent the solution is. The air-dried films are then placed in a small Petri dish with the specimen side up. The film is now covered with 10 to 15 drops of the alcoholic staining mixture for ½ minute. The preparation is thus fixed and the staining is begun. Add enough distilled water to cover the specimen (usually 10 to 15 c.c.), and agitate the dish till a homogeneous mixture of the stain is secured. Allow the specimen to remain in this mixture 5 minutes. The film is now washed in distilled water, dried, and mounted in balsam. The spirochetes are stained pink.

3. *Stern's Silver Impregnation Method.*[102]—The smears of serum, air dried, are first placed in the incubator at 37° C. for a few hours. They are then transferred to a colorless glass filled with 10 per cent

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[100] From Mallory, F. B., and Wright, J. H. *Pathological Technique.* Philadelphia and London, 5th edition, 1911, p. 418.

[101] Giemsa, G. "Ueber eine neue Schnellfärbung mit meiner Azur-eosinlösung." *München. med. Wchnschr.*, 1910, LVII, 2476.

[102] Stern, M. "Ueber den Nachweis der Spirochæta pallida im Ausstrich mittelst der Silbermethode." *Berlin. klin. Wchnschr.*, 1907, XLIV, 400.

aqueous solution of silver nitrate, and exposed to diffuse daylight for several hours. The preparation gradually assumes a brown color. When this has reached a certain shade (quickly learned by practice) and the film shows a metallic luster, it is removed from the silver and washed in distilled water. In a properly treated specimen the spirochetes are stained deep black on a pale brown or colorless background. The organisms are slightly thicker than in specimens stained with Giemsa's stain. Anomalies in staining may be encountered. At times the spirals are more deeply stained at the upper bend of the curve than at the lower, which then appears gray. Or there may be only a row of deep black granules or dots representing a spirochete. The erythrocytes are well preserved, show a delicate, black contour, and present a number of fine granules.

The specimen should not be exposed to direct sunlight while it remains in the silver, for, though the preparation quickly becomes dark and even black, the spirochetes are unstained.

4. *Fontana's Method of Staining Spirochetes.*[103]—A thin film of the material to be examined, as free from blood as possible, is spread on a slide and allowed to dry. Cover repeatedly for about a minute with the following solution (Huge's):

Acetic acid, glacial	1.0
Formaldehyd, 40 per cent	2.0
Distilled water	100.0

To complete fixation, alcohol is dropped on the slide and then flamed. The following mordant is then applied:

Tannic acid	5.0
Distilled water	100.0

Warm gently till steam rises and allow the mordant to remain on the specimen one-half minute longer.

Wash the slide in running water a few seconds and then cover the film with Fontana's solution, prepared as follows:

Silver nitrate 5 per cent solution is prepared. To this add ammonia drop by drop from a capillary pipette, till a sepia brown precipitate forms and redissolves. To this solution more silver nitrate is added till a solution is produced which remains slightly cloudy on shaking.

[103] From Stitt's *Bacteriology, Blood Work and Parasitology,* Phila., 1920.

The Fontana's solution on the film is warmed gently till it steams, and is then allowed to act one-half minute longer.

Spirochetes appear dark brown to black, but fade in a few days in cedar oil or Canada balsam.

5. *Burri's India Ink Method.*[104]—One loopful of serum is mixed on a glass slide with a loopful of India ink, and spread in a thin film by means of a second slide. The slides must be perfectly clean. The film, which is allowed to dry in the air, should be dark brown or black. A drop of immersion oil is placed on the specimen, which is ready for examination. Bacteria, spirochetes, blood corpuscles, etc., are unstained, and appear as refractive bodies on the dark background. According to Cohn and others, the best results are obtained with Gunther-Wagner's Chin-Chin black pearl ink, though fair success may be had with other inks, as Carter's or Higgin's.

6. *Dark Field Illumination.*—The simplest and quickest method of demonstrating *S. pallida* is by means of the dark field condenser. The technic is readily acquired and the cost of the apparatus is fairly reasonable. The living, motile spirochetes are easily detected in serum obtained from syphilitic lesions. Avoidance of blood in the specimen for examination is important, as the cells, if numerous, obscure the field. If the cells are not too numerous, dilution with pleural or ascitic fluid at times makes it possible to use a specimen which would otherwise have to be discarded.

Treponema pertenue[105] (Synonyms: *Spirochæta pertenuis, Spirochæta pallidula*).—*Treponema pertenue,* discovered by Castellani, is the causative agent of *yaws* (*framboesia*), and was first found in yaws papules. Castellani states that it "is an extremely delicate, spiral-shaped organism, varying in length from a few micra to 18 or 20 micra or even more. It is very slender. It does not stain easily, but good results may be obtained with Giemsa's method, and also with Leishman's stain, provided the alcoholic solution is allowed to act for five minutes, and the subsequent mixture with distilled water from one-half to several hours. Using either of these methods, the *Treponemata* stain purplish. The number of coils varies from 6 to 20 or more."

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[104] Cohn, J. S. "On the means of finding the Spirochæta pallida with special reference to the India ink method." *Interstate Med. Jour.,* 1911, XVIII, 26.

[105] For an excellent discussion of yaws, see Moss, W. L. and Bigelow, G. H. "Yaws: An analysis of 1046 cases." *Trans. Assoc. Amer. Phys.,* 1921, XXXVI, 105.

Bacillus Tuberculosis.—The *Bacillus tuberculosis* (Pl. V, *E*) is not easily recognized in the urine because of the constant presence of the smegma bacillus on the genitalia, an organism whose morphology is quite similar to that of the tubercle bacillus, and which cannot be separated from the latter with certainty by staining. It is, therefore, necessary to exclude the smegma bacillus from the urine as a preliminary step in the examination for the tubercle bacillus, as Young and Churchman [106] have shown. The technic which these authors have developed and which has proved reliable is as follows: The foreskin, if present, is rolled back and the glans penis is washed thoroughly with green soap and water, using large amounts of water for the rinsing. The irrigating catheter is now introduced about six inches into the urethra (to the triangular ligament), while the patient keeps the sphincter urethræ closed to prevent fluid entering the bladder. About one quart of sterile water is employed in the irrigation of the urethra. Since the smegma bacillus is not found back of the triangular ligament, the urinary tract is practically freed of this organism by the procedure just described. The patient is now instructed to urinate into three glasses, and a portion of the urine from the third glass is centrifugalized at least five minutes at high speed. Three smears of the sediment obtained are made and stained for tubercle bacilli by the Ziehl-Neelsen method (p. 211). If a thorough examination of the stained specimens reveals no acid-fast bacilli, the result of this particular examination is reported as negative.

Occasionally pus is so abundant that the search for tubercle bacilli is very difficult. When such is the case the antiformin method may be resorted to (p. 212). The pus cells are completely dissolved. It must be remembered that all acid-fast bacteria are resistant to the action of antiformin, so that it is necessary to observe the usual precautions to exclude the smegma bacillus.

Actinomyces bovis has been encountered in the urine in twelve cases, chiefly in connection with pyelonephritis. It should be sought in cases of suspected tuberculosis of the kidney, in which no acid fast bacilli are demonstrable.[107]

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[106] Young, H. H., and Churchman, J. W. "The possibility of avoiding confusion by the smegma bacillus in the diagnosis of urinary and genital tuberculosis." *Amer. Jour. Med. Sci.*, 1905, CXXX, 52.

[107] Cecil, H. L. and Hill, J. H. "Actinomycosis of the urinary organs: Report of a case of pyelonephritis in which *Actinomyces bovis* was found." *Jour. A. M. A.*, 1922, LXXVIII, 575.

ANIMAL PARASITES IN THE URINARY PASSAGES

1. **Trichomonas Vaginalis.**—*Trichomonas vaginalis,*[108] a flagellate closely allied to and possibly identical with, *Trichomonas intestinalis,* may be found in the vagina and occasionally in the bladder. In either locality it comes in contact with the urine, in which it may be found. It thrives only in an acid medium; this is supplied by the normal vagina, except during the menstrual period, when the mucosa is bathed in the bloody discharge. Trichomonas vaginalis is a pear-shaped organism with pointed extremity and, at the anterior rounded end, presents four flagella. An undulating membrane is also present. It measures usually 0.015 to 0.022 mm. in length and 0.010 to 0.015 mm. in width, though larger and smaller forms occur. It appears to be a specific parasite of the female sex, and is non-pathogenic.

2. **Filaria Bancrofti.**—*Filaria bancrofti* (Synonyms: *F. nocturna, F. sanguinis hominis*) is of common occurrence in tropical and subtropical countries; an endemic center exists in South Carolina. Its embryos may be found in the urine in cases of parasitic hematochyluria. They are either free in the urine and actively motile in a fresh specimen, or caught in the clot. The urine usually has a milky appearance (chyluria), but may have a pinkish color from the admixture of blood. Semitransparent clots may form in the urine. The urine passed at night is usually much more turbid and milky than that passed in the day, which is at times quite clear. Under the microscope, no fat droplets are seen, as a rule, as the fat is emulsified; leukocytes, chiefly lymphocytes, and a variable number of red cells are found, and often *Microfilariae* and crystals of calcium oxalate.[108] The fat must be extracted with ether before the urine may be tested for albumin, which is always present, owing to the admixture of lymph, and often of blood. If there is no appreciable quantity of fat in the urine, the term lymphuria is used in place of chyluria. The same patient may have at times attacks of chyluria and hematochyluria, and at other times attacks of lymphuria and hematolymphuria. The embryos are 0.290 to 0.320 mm. long, with a thickness of 0.0075 to 0.0084 mm. Wherry and McDill[109] found

[108] Dock, G. "Trichomonas as a parasite of man." *Amer. Jour. Med. Sci.,* 1896, CXXXI, 1.

[108a] Castellani, A. and Chalmers, A. J. *Manual of Trop. Med.,* N. Y., 3d ed., 1919, p. 635; 1608.

[109] Wherry, Wm. B. and McDill, J. R. "Notes on a case of hematochyluria, together with some observations on the morphology of the embryo nematode-Filaria nocturna." *Jour. Infect. Dis.,* 1905, II, 412.

the average of four measurements in their case 0.327 by 0.0074 mm. (Fig. 87, page 350).

3. **Dioctophyme Renale.**—*Dioctophyme renale* (*Eustrongylus gigas*), another nematode, is excessively rare in man, though not very uncommon in dogs in this country. It is the largest round-worm parasitic in man. Its habitat is the kidney. Lodging in the pelvis of the kidney, it produces a pressure atrophy until, when the parasite reaches maturity, little or

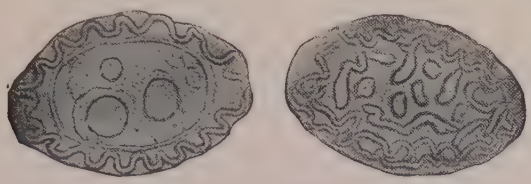

Fig. 19.—Ova of Dioctophyme Renale. ×400 (after Emerson).

none of the parenchyma of the kidney remains. The male measures 14 to 35 cm. in length, with a thickness of 0.4 to 0.6 cm. The female is much larger—25 to 100 cm. long and 0.4 to 1.2 cm. thick, and is bright red in color. Infection is diagnosed by finding the ova (Fig. 19) in the urine. The latter are oval, 0.064 to 0.068 mm. in their long axis by 0.042 to 0.044 mm. in the short (Blanchard). The shell is covered with an albuminous coating, which is stained brown and is thrown into ridges, making the surface of the egg appear more uneven than that of

Fig. 20.—Ovum of Schistosoma hæmatobium (after Looss, from Stitt).

Ascaris lumbricoides, which it resembles somewhat. The albuminous coating is lacking at the poles of the ovum and the latter appear colorless.

4. **Schistosoma Hematobium.**—*Schistosoma hematobium* (*Bilharzia hæmatobia*),[110] a trematode, is an important urinary parasite in tropical

[110] Lane, C. G. "Bilharziasis; report of a case with appendicitis; literature since 1904." *Boston Med. and Surg. Jour.*, 1911, CLXIII, 937. (Note.—At the time this paper was published, *S. mansoni* had not been established as a species. The literature, therefore, includes *S. hematobium* and *S. mansoni* under the designation *S. hematobium* or *Bilharzia hematobia.* R. S. M.)

and subtropical climates. It is especially prevalent in Egypt. The parasite lives in the veins of the urinary bladder; it deposits its ova in the veins. The ova then pass from the veins to the bladder. The ova are similar to those found in the feces (q. v.), except for the fact that they are a little smaller and that the spine is terminal instead of subterminal (Fig. 20). The ova measure 0.046 to 0.050 by 0.110 to 0.120 mm. (Castellani and Chalmers). As the sharp-spined ova pierce the wall of the vein, hemorrhage, of course, ensues, with the result that hematuria is a quite constant symptom of the infection.

5. **Echinococcus Disease.**—In echinococcus disease of the kidneys or of the urinary tract, the contents of an echinococcus cyst may be voided. Thus, the hooklets (Fig. 60, p. 224) may be found in the sediment or even a daughter-cyst may be voided (Sahli).

PROSTATIC FLUID

Prostatic fluid [111] is obtained by massage of the prostate gland per rectum, the urethra having been irrigated previously. The amount of fluid obtained at a "milking" varies from a few drops to 4 or 5 c.c. The fluid is of low specific gravity, slightly tenacious, grayish-white, yellowish, or greenish in color, and usually has a milky turbidity from the lecithin granules contained in it.

A fresh drop of the fluid is examined microscopically for the presence of motile *spermatozoa*. *Lecithin* granules vary considerably in size. The smallest are minute specks, the largest four micra or more in diameter. They are moderately refractive. *Corpora amylacea,* laminated bodies with a granular center, may be met with, especially in specimens obtained from the aged. They resemble starch granules not only in form, but also in the fact that they may be stained blue with iodin. Various kinds of epithelial cells may be found. In examining for *epithelial and pus cells* it is well to add dilute acetic acid to bring out the cell nuclei. *Spermin crystals* (Böttcher's crystals), transparent needles or whetstones, are observed at times. They may resemble Charcot-Leyden crystals, but differ from the latter in that they are soluble in alkalies and in formaldehyd.

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[111] From Emerson, C. P. "Clinical Diagnosis."

FUNCTIONAL DIAGNOSIS OF THE KIDNEY

Many tests, some simple, others complicated, have been introduced to measure the functional capacity of the kidneys. All have had certain well-recognized limitations, and none has been particularly helpful where the two kidneys are equally involved in the disease process, as in nephritis, until Rowntree and Geraghty described their phenolsulphonephthalein test. This constitutes by far the most satisfactory and exact method of functional diagnosis, and, in the hands of a large number of workers, has proved of immense value in the diagnosis, prognosis, and treatment of both medical and surgical diseases of the kidneys. In surgical affections some of the simpler tests may be used in conjunction with the "phthalein" test. The specimens obtained by ureteral catheterization often permit of accurate diagnostic conclusions through comparison of the microscopic and chemical findings from each kidney. Urea determinations with the hypobromite method are frequently made to advantage; for, though the values obtained represent total nitrogen more nearly than urea, nevertheless the comparative efficiency of the two kidneys may be fairly accurately determined in many instances. The information thus gained is practically always corroborated by the phthalein test, but frequently the latter will give evidence of disease when other tests are misleading. In nephritis and analogous conditions, where each kidney is involved to about the same extent, the phthalein test is the only reliable measure of functional capacity, aside from blood chemical analyses.

The Phthalein Test of Rowntree and Geraghty.[112]—Twenty to 30 minutes before starting the test the patient is given 300 to 400 c.c. of water to insure free urinary secretion. Then the bladder is catheterized with aseptic technic, and 1 c.c. of a solution containing 6 mg. of phenolsulphonephthalein[113] is administered intramuscularly into the lumbar muscles. (The solution is prepared as follows: "0.6 gm. of phenolsulphonephthalein and 0.84 c.c. of $\frac{2}{N}$ sodium hydrate are diluted with 0.75 per cent sodium chlorid solution up to 100 c.c. This gives

[112] Rowntree, L. G., and Geraghty, J. T. (a) "An experimental and clinical study of the functional activity of the kidneys by means of phenolsuphonephthalein." *Jour. Pharm. & Exp. Therap.*, 1910, I, 579. (b) "The sulphonephthalein test for estimating renal function." *Jour. A. M. A.*, 1911, LVII, 811. (c) "The phthalein test." *Arch. Int. Med.*, 1912, IX, 284.

[113] The substance is supplied by Hynson, Westcott & Co., Charles and Franklin Sts., Baltimore, Md. It is dispensed in glass ampuls. The dose is 1 c.c.

the monosodium or acid salt, which is red in color, and which is slightly irritant locally when injected. It is necessary, therefore, to add 0.15 c.c. more of the $\frac{2}{N}$ hydroxid, a quantity sufficient to change the color to a beautiful Bordeaux red. This preparation is non-irritant.")

The catheter is retained until the dye appears in the urine, when it may be withdrawn if there be no obstruction, as from enlargement of the prostate. The urine is collected in a vessel, which contains one drop of 25 per cent sodium hydrate, since the red color of the drug is apparent only when the reaction of the solution is alkaline. The time of appearance of the dye in the urine is noted. At the end of the first hour after administering the phthalein the patient urinates into a clean receptacle, and into a second receptacle at the end of the second hour. Each specimen is now rendered distinctly alkaline by the addition of 25 per cent sodium hydrate in order to elicit the maximal color. The dye is yellow or orange in an acid urine, but becomes purplish-red when the reaction is alkaline. Place the urine (each specimen separately) in a volumetric flask of 1,000 c.c. capacity, and add distilled water to 1,000 c.c. Mix thoroughly, and filter a small portion for comparison with the standard solution. Each is examined colorimetrically for phthalein content. The sum of the determinations indicates the total function.

The standard solution is an aqueous solution of phenolsulphonephthalein containing 6 mg. to the liter, as described above, the solution being rendered strongly alkaline. *Colorimetric determination* of the quantity of drug excreted in a given specimen is made with the Duboscq colorimeter or with Rowntree and Geraghty's modification of the Autenrieth-Königsberger colorimeter.[114] Employing the Autenrieth-Königsberger instrument (Fig. 21), the standard solution is placed in the wedge-shaped glass. A filtered portion of the urine, rendered alkaline and diluted to one liter, as described, is poured into the rectangular glass. In one side of the case of the instrument there is a narrow slit, the opposite side being frosted glass. With the frosted glass held to the light, the observer looks through the slit and sees the two columns of fluid—urine and standard solution. By means of a thumb-screw, the

[114] This instrument costs about one-fifth as much as the Duboscq colorimeter and is perfectly satisfactory. The Rowntree and Geraghty modification may be had from Hynson, Westcott & Co., Baltimore, Md. It is made by Hellige in Freiburg.

wedge containing the standard solution is elevated or lowered until the color intensity is alike on the two sides. The percentage of coloring matter in the urine is now read directly from the position of the indicator on the scale.

Test Tube Colorimeter.—A simple and very satisfactory *colorimeter* may be made by anyone, at almost no cost. A standard solution is prepared by diluting 1 c.c. of "phthalein" (0.6 mg. phthalein in 1 c.c.)

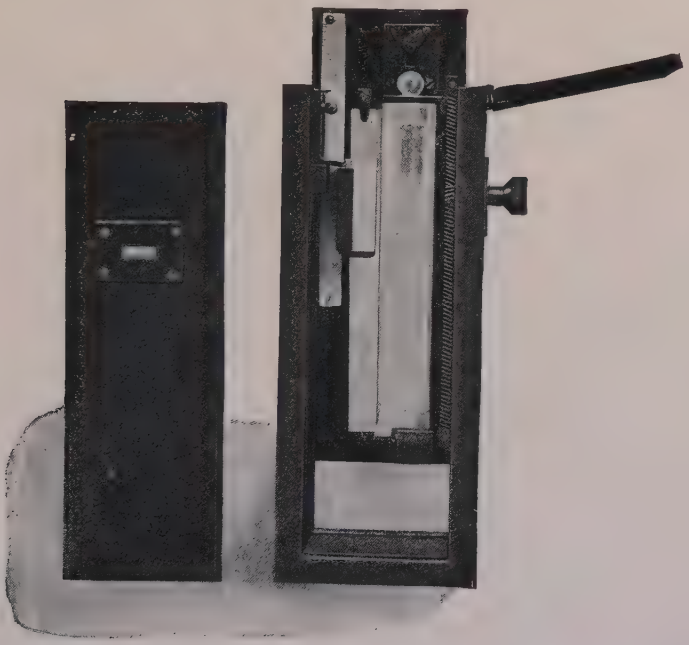

FIG. 21.—THE AUTENRIETH-KÖNIGSBERGER COLORIMETER AS MODIFIED BY ROWNTREE AND GERAGHTY FOR THE DETERMINATION OF PHENOLSULPHONEPHTHALEIN.

to one liter with distilled water. Before the full amount of water has been used, add sufficient 25 per cent sodium hydroxid to render the solution distinctly alkaline, in order to bring out the maximal color of the phthalein. Next place ten test tubes of *uniform diameter* in a rack, numbering the tubes consecutively 10, 9, 8, etc., to 1, or if it is desired to represent percentage, 100, 90, 80, etc., to 10. Into the first tube place 10 c.c. of the standard solution just prepared; into the second tube 9 c.c. of the standard solution and 1 c.c. of distilled water, to

which the same quantity of alkali per liter has been added, as was used when making the standard solution; the third tube (8 or 80) receives 8 c.c. of standard solution and 2 c.c. of alkalinized distilled water. Thus, the amount of standard solution is decreased by 1 c.c., that of the alkalinized distilled water increased by 1 c.c. in each successive tube, until the last tube receives 1 c.c. of standard solution and 9 c.c. of alkalinized distilled water. The tubes are now tightly stoppered; it is well to seal them with paraffin to be certain there is no evaporation. Thus, a series of tubes with decreasing proportions of phenolsulphonephthalein is obtained, the tubes in order representing 100 per cent, 90 per cent, 80 per cent, and so on, to 20 per cent and 10 per cent solutions. Two additional empty tubes, of diameter uniform with those containing the standard solutions, are needed for the solutions (urine) to be tested. After diluting the urine as already described, 10 c.c. are placed in a test tube and the color is compared with the standard solutions. Readings accurate enough for all practical purposes may be obtained.

The standard solutions, when not in use, should be kept in a dark place. The solutions keep well, but it is advisable to check them at intervals of one to two months by comparison with a freshly prepared standard solution. When they begin to fade, it is the work of only a few minutes to prepare fresh standard solutions.

As a routine procedure Rowntree and Geraghty recommend intramuscular (lumbar) injection of the drug. They employ a Record syringe of 2 c.c. capacity graduated in $\frac{1}{5}$ c.c. "Whereas in the case of phthalein a normal kidney excretes the greater part of the dye injected within two hours of the time of its administration, and then only a small trace for the next two hours, the moderately diseased kidney secretes a fair amount within the first two hours, say 50 per cent of that excreted by the normal kidney, but, the concentration in the blood still being high, it continues to excrete a fair amount in the following two hours, so that at the end of four hours little difference may exist in the total work accomplished. One-hour and, at the most, two-hour observations are, therefore, recommended. In cases in which only slight changes in function exist this can be most accurately demonstrated by one-hour collection following the use of an intramuscular (lumbar) injection." With intravenous injection the time of appearance and the duration of maximal elimination are shortened, but the results are, on the whole, less trustworthy.

With *normal* kidneys the following findings have been obtained:

Administration	*Time of Appearance*	*Quantity Excreted*
Intramuscular (lumbar)	5-11 min.	51.8-64.1% first hour
"	" "	60-85% two hours
Intravenous	3-5 "	34-45% in 1st 15 min.
" "	" "	50-65% in 1st 30 min.
"	" "	63-80% in 1st 60 min.

As the intensity of color of the dye gradually diminishes in alkaline urine, it is necessary that the determinations be made within a few hours at the most. If the estimation of the dye must be delayed for some hours or days, the urine should be rendered distinctly acid, as the phthalein remains unchanged in acid solution. Just before making the colorimetric determination an excess of alkali is then added to elicit the full strength of the color.

When urine is highly pigmented, error in the colorimetric readings may be lessened by making up a standard solution containing urine. The error from this source is, however, so small as to be negligible in most instances.

When the phthalein is being *collected* from the kidneys separately *by means of ureteral catheters*, the time of appearance of the dye is noted and the catheters are then left in place for 20 to 30 minutes; the dye should be given *intravenously*. The *normal* excretion for *each* kidney is about 1 per cent per minute for the first 20 minutes and about ½ per cent per minute for the next 10 minutes; or 22 to 25 per cent for *each* kidney for 30 minutes.

Bilateral Renal Disease.—The phthalein output is decreased in:

1. *Nephritis.*—The amount of the dye excreted often is entirely independent of the quantity of water eliminated, though in some cases the excretion is greater with free diuresis.[115]
   a. Acute nephritis.—Usually a marked decrease, once the disease is well established, with increasing amounts eliminated as recovery takes place.
   b. Chronic parenchymatous nephritis.—Variable. In some cases marked decrease, even to zero, as in a recent case. (With

[115] Snowden, R. R. "Variation of the phenolsulphonephthalein excretion with the urine volume in chronic interstitial nephritis." *Arch. Int. Med.*, 1921, XXVIII, 603.

general anasarca, employ intravenous administration of the phthalein, since uncertainty may arise in regard to the rate of absorption after intramuscular injection). At times, the excretion is normal or above normal.[116]

c. Chronic interstitial nephritis.—The more advanced the disease, the more marked the decrease in excretion of phthalein, until, in the final stages, the dye fails to pass through the kidneys in appreciable amounts. In the last group, as Rowntree and Geraghty[117] have shown, the prognosis is very bad, death usually ensuing within two months.

Nephritides in general usually show a reversal of the normal, in that the amount of dye excreted in the second hour is often greater than that excreted in the first hour.

2. *Pyelonephritis.*
3. *Hydronephrosis.*
4. *Renal tuberculosis.*
5. *Polycystic kidneys.*

In these diseases (2 to 5), the output of phthalein is usually decreased in proportion to the degree of renal damage.[118]

6. *Chronic passive congestion.*—In chronic passive congestion, usually occurring in connection with chronic cardio-renal disease or with cardiac insufficiency, the phthalein output is often markedly diminished. As the circulation in general, and that through the kidneys in particular, improves, there is a rise in the phthalein output. If the output remains subnormal after complete reëstablishment of the circulation, permanent damage to the kidneys is indicated.
7. *Hypertrophy of the prostate, with obstruction, residual urine, etc.*—The phthalein output will indicate the degree of renal damage, where the usual urine examinations and physical examinations may fail. Employment of the test has greatly lessened the mortality from operations on the prostate.[119] In patients with much residual urine and a marked decrease in phthalein output, the operative risk is great; the use of a retention catheter often results

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[116] Baetjer, W. "Superpermeability in nephritis." *Ibid.*, 1913, XI, 593.
[117] Rowntree, L. G. and Geraghty, J. T. *Loc cit.*, p. 120.
[118] Rowntree, L. G. Personal communication.
[119] Young, H. H., and Frontz, W. A. "Preliminary treatment for prostatectomy in unfavorable cases." *Jour. A. M. A.*, 1917, LXVIII, 526.

in marked increase in output, and greatly lessens the risk from operation.

Unilateral Renal Disease.[120]—The urine is collected by ureteral catheterization. The amount of dye excreted in one-half hour after intravenous administration is determined separately for each kidney. The results indicate, with a fair degree of accuracy, the relative efficiency of the two kidneys. It is to be remembered that, with marked unilateral disease (tuberculosis, calculus, pyonephrosis, etc.), the combined amount excreted by the two kidneys may be practically normal. Delay in the appearance of the dye from one kidney also usually indicates the more diseased side. (Normally, the dye appears on the two sides at the same time or within five minutes.) The *normal* output for each kidney is 22 to 25 per cent for 30 minutes (see page 117).

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[120] For an excellent discussion of functional renal diagnosis, see papers by H. Berglund; W. H. Olmsted and J. R. Caulk; W. A. Frontz and J. T. Geraghty; J. B. Squier, C. G. Bandler and V. C. Myers; B. A. Thomas. *Jour. A. M. A.*, 1922, LXXIX, 1375-1391.

CHAPTER II

THE GASTRIC JUICE

The gastric juice is obtained for analysis with the stomach tube, following the administration of a test breakfast or meal. The test breakfasts or meals are employed for the sake of simplicity and to obtain comparable conditions. It is because of the many unknown factors involved, such as the quality of food, the length of time it has remained in the stomach, the condition of the stomach before the food was taken, etc., that little dependence can be placed on the results of analysis of vomitus.

Test Breakfasts.

1. *Ewald's breakfast* consists of 40 gm. of bread and 400 c.c. of water or weak tea without sugar or cream.

2. *Dock's breakfast* is the same as the Ewald breakfast, except for the substitution of one shredded wheat biscuit for the bread.

3. *Boas' breakfast* is prepared by boiling one tablespoonful of oatmeal in 800 c.c. of water till the volume equals about 400 c.c.

In this country Dock's breakfast is rapidly coming into use. This and the Boas breakfast possess a certain advantage over the Ewald breakfast, in that no lactic acid is contained in the food, a possible source of error when bread is used. Any of the breakfasts is allowed to remain in the stomach *one hour,* as a rule, at the end of which the stomach tube is introduced and the gastric contents evacuated. With normal gastric motility the stomach yields 20 to 50 c.c. one hour after a test breakfast (Boas). To eliminate the possibility of error through giving the breakfast to a patient whose stomach contains part of the previous meal, lavage may precede the breakfast, being performed preferably an hour or so before giving the breakfast.

In using the test breakfasts, misinterpretation may follow, if conclusions are drawn from the results of a single meal. In gastric neuroses, for example, there may be a hyperacidity one day, normal acidity the next day, and even an anacidity the third (so-called heterochylia). It

is advantageous from every standpoint to repeat the breakfast on *three successive mornings,* a plan adopted in Dock's clinic at the University of Michigan more than twenty years ago. Furthermore, it is desirable, and necessary, to repeat the tests from time to time, to control the effects of treatment. Fractional analysis is not reliable.

4. The *Fischer meal* consists of an Ewald or Dock breakfast with three-quarters of a pound of finely chopped, lean beef, broiled and slightly seasoned. This meal, like the Riegel dinner, excites the secretion of hydrochloric acid better than the breakfasts. It is usually allowed to remain in the stomach three hours.

5. The *Riegel dinner* is more appetizing than any of the preceding meals. It is composed of:

One plate of meat broth.

Beefsteak weighing 150 to 200 gm. (5 to 7 oz.).

Mashed potatoes, 50 gm. (1½ oz.).

One roll.

Riegel says:[1] "As a rule, I empty the stomach four hours after the meal, provided that other indications are not present that determine me to select some other time. If the stomach is found empty after four hours, I know that the motor power of the organ is good; no conclusions, however, can be drawn in regard to its peptic powers. If the stomach is found empty after four hours, its contents should be withdrawn earlier the next day; if, on the other hand, a large quantity of coarse and only half digested morsels of food are found after four hours, the examination on the next day should be made later. A single examination is never permissible." The dinner is usually given at the time of the midday meal.

Other test meals have been proposed but are not very generally employed for purposes of gastric analysis.[2]

EXAMINATION OF THE FASTING STOMACH

As the examination of the fasting stomach should precede test meals, the results obtained may be considered before passing to the examination of the gastric contents.

The normal stomach empties itself in about seven hours. Passage of the stomach tube before breakfast should, therefore, lead to the

[1] Riegel, F. *Diseases of the Stomach* (edited by C. G. Stockton). "Nothnagel's Practice." Philadelphia and London, 1905, pp. 79 *et seq.*

[2] Riegel, F. *Loc. cit.*

recovery of little fluid or none at all. *Normally,* the amount rarely exceeds 50 c.c. (Emerson). When 100 c.c. or more are obtained, there exists either a gastro-succorrhea (continuous secretion) or retention of the gastric contents (Boas). Normally or with hypersecretion, swallowed saliva or sputum may be seen in the fluid. With retention, food eaten the previous evening or several days before may be recognized; this should always be looked for, as it furnishes conclusive evidence of stagnation. The ease with which the food may be recognized will depend upon two factors: (1) the chemical composition of the gastric secretion, and (2) the nature of the food. With good acidity, proteins may be well digested, whereas with a deficiency of acid they are little altered. Parts of food which resist the action of the gastric juice, such as the seeds of small fruit or berries, are easily detected. In fact, when defective motor power is suspected, it is a useful procedure to give raspberry jam or some similar preparation in the evening, and look for the seeds in the gastric contents or lavage the following morning. At times excessive quantities of fluid are found in the fasting stomach. The *normal* organ has a capacity of about 1,600 c.c. (Ewald); a stomach which can retain more than this quantity is dilated.

In addition to the points just enumerated, the fluid obtained from the fasting stomach should be subjected to the examination to be described for the gastric contents.

MACROSCOPIC EXAMINATION OF THE GASTRIC CONTENTS

Quantity.—In the examination of the gastric contents obtained one hour after a test breakfast, the quantity of fluid recovered is measured. Boas finds that the amount usually lies between 20 and 50 c.c. with *normal* gastric motility. Higher amounts, however, are certainly obtained in health at times; 80 c.c. is not unusual. When 150 to 200 c.c. are found in the stomach, hypomotility is quite definitely indicated. A stomach which is repeatedly found empty one hour after a test breakfast has hypermotility, and it is then necessary to remove the contents after three-quarters or one-half hour.

Odor.—The normal gastric contents are practically odorless. In disease the odor may be sour or rancid (acetic acid, butyric acid, etc.), putrid, fecal, etc. The odor of drugs should also be looked for.

Mucus.—The presence of an excess of mucus is most easily detected by pouring the gastric juice from one receptacle to another. If the

amount be abnormal, the condition is at once recognized. Mucus from the respiratory passages floats because of the bubbles contained in it. From the pharynx and esophagus there may be a considerable quantity of mucus secreted during the passage of the stomach tube. It runs out along the side of the tube, and is not aspirated through the tube, as in the case of true gastric mucus or swallowed sputum.

Color.—*Normally* the gastric secretion is practically colorless. The regurgitation of *bile* from the duodenum may impart a deep yellow or green color, the intensity depending on the relative proportion of bile. Blood, when fresh, is characteristic in appearance; if it has remained in the stomach long enough to undergo change, the bright red color is lost, and is replaced by a dark brown, producing in many instances the so-called "coffee-ground" appearance. The color of the gastric juice may also be altered by food or drugs.

Food.—The state of digestion of the food is of great importance. After the usual test breakfasts, carbohydrate forms the bulk of the food ingested. The alterations found are due chiefly to ptyalin of the saliva. With hyperacidity this enzyme is quickly destroyed, with a consequent inhibition or arrest of amylolysis. After a mixed meal, such as the Riegel dinner, more information may be gained by inspection of the gastric contents. The appearances are well described by Riegel.[3] "In some cases a very fine, uniform, mushy liquid mass is seen that contains no coarse elements at all; in others, again, a mass of food containing many coarse pieces of meat that look as if they had just been swallowed; in addition, there is frequently an abundant admixture of mucus. In some cases there is so much mucus that the food looks like a tough mass and passes through the sound with difficulty, and is very difficult to filter. In other cases there is a large quantity of fluid contents that forms three layers when kept in a glass vessel; at the bottom is seen a layer consisting of fine remnants of amylaceous material; above this a large layer of cloudy fluid, and on the top a foamy layer. If the latter is present it may be considered evidence of gaseous fermentation. This consistency of the stomach contents is found chiefly in cases in which there is stagnation or in which there is motor insufficiency . . . usually in cases in which there is an abundant quantity of free hydrochloric acid. . . . If the food remnants obtained from the stomach in different diseases are compared, the great significance of macroscopic inspection

[3] Riegel, F. *Loc. cit.*, p. 86.

will be understood. In many instances this method alone will give us diagnostic points which we would otherwise obtain only by complicated chemical examinations. There are cases, for instance, in which the stomach contents do not give any of the reactions for free hydrochloric acid. This shows that there is a deficit in the stomach. Sometimes, however, when free hydrochloric acid is absent, we find only a relatively small amount of finely distributed food residue; at other times we may see larger quantities of coarse food particles. If we limit ourselves to examining the filtrate in both these cases for free hydrochloric acid, we will probably consider that the two are alike, and, as a matter of fact, they are alike in regard to their free hydrochloric acid, for in neither do we see a formation of free hydrochloric acid. If, however, we consider the quantity and the appearance of the stomach contents in both, we shall see that in the first case the peptic power is better than in the second. The first case is functionally nearly normal, for all the albumin has been digested; at the same time there was no residue of free hydrochloric acid. In the second case it is different; here the production of acid was subnormal, as shown by the disturbed digestion of meat. If this case is more carefully examined, it will be found that the deficit of hydrochloric acid is large, whereas in the first case it is small. In this way macroscopic examination frequently gives us a clear picture of disturbances of function. . . ."

The careful macroscopic analysis of the gastric contents, it is evident, is of the greatest value.

CHEMICAL EXAMINATION OF THE GASTRIC CONTENTS

Reaction.—The reaction of the gastric contents is tested with litmus paper. It is usually acid. An alkaline or neutral fluid may be obtained.

Hydrochloric Acid

Hydrochloric acid is the most important chemical constituent of the gastric juice from the clinical standpoint. *Normally,* it is present in excess, that is, a test for free hydrochloric acid is always obtained.

Qualitative Tests for Free Hydrochloric Acid

1. **Von den Velden's Methyl Violet Test.**—Add a few drops of a saturated aqueous solution of methyl violet to a test tube nearly filled with water. The dilute solution of the dye should be transparent and

violet or purple in color. It is divided equally in two test tubes. To the one add an equal quantity or less of gastric juice, to the other an equal volume of water. Free hydrochloric acid is indicated by a change in color from violet to blue, the portion to which water alone is added serving as a control. The test is said to indicate 0.025 per cent of free hydrochloric acid. According to Riegel, the test is especially valuable, since, when it is positive, it means that there is sufficient free acid for protein digestion.

A second method of performing the test, which is useful when the amount of gastric juice at one's disposal is small, consists in spreading a thin layer of the dilute methyl violet solution in a porcelain plate, and then placing a drop of gastric juice in contact with it. Where the two fluids run together, the violet color is changed to blue in the presence of free acid.

Lactic acid does not interfere with the methyl violet reaction, since it is given only by 0.4 per cent or stronger solutions, which never occur in the stomach.

2. **Günzberg's Test.**—This is the standard test for free hydrochloric acid. It is positive only in the presence of a free mineral acid.

Reagent:

Phloroglucin	2.0 gm.
Vanillin	1.0 gm.
Alcohol, absolute	30.0 c.c.

Dissolve and keep in a brown bottle, tightly stoppered. As the reagent does not keep well, it is advisable to make small quantities, so that it may be renewed every few months. It is well to test the reagent from time to time with dilute hydrochloric acid to prove its reliability.

A few drops of the reagent are evaporated to dryness in a porcelain dish by warming gently over a Bunsen burner. A drop of gastric contents is brought in contact with the yellowish-brown stain left by the reagent, and is evaporated. If free hydrochloric acid is present, an intense red color develops, where the reagent and gastric juice have mixed. Instead of evaporating the reagent and gastric juice separately, equal quantities of the two may be mixed (one or two drops of each) and evaporated, when the color change appears.

The evaporation must be performed with great care. It is easy to burn the reagent by overheating; the test then fails, even though there be an abundance of free acid present. The degree of heat may be tested by touching the bottom of the porcelain dish with the finger. The dish is held in the flame a second, removed, tested; this procedure, repeated at intervals, accomplishes the desired result with a little practice. Blowing on the specimen when it is removed from the flame hastens the evaporation, and at the same time lowers the temperature. A safer method of evaporation is the use of a water bath.

The test is sensitive to free hydrochloric acid in 0.01 per cent solution. It is specific in the sense that a positive reaction is only obtained with free mineral acid; organic acids do not give the test.

3. **Tropeolin Test.**—A saturated alcoholic solution of tropeolin 00 is prepared. Three to four drops of this reagent and a like quantity of the gastric juice are spread over the surface of a porcelain dish, and carefully evaporated to dryness. In the presence of free acid the color becomes violet or blue. The test is less sensitive than either of the preceding tests. It is positive with free hydrochloric acid in a dilution of 0.03 per cent. Lactic acid solutions of 0.24 per cent or stronger give the reaction (Ewald); in the stomach it is doubtful whether lactic acid ever occurs in sufficient concentration to give the test.

In place of the concentrated alcoholic solution of tropeolin 00, Riegel recommends a saturated aqueous solution.

4. **Congo-paper Test.**—Filter paper is saturated with a concentrated aqueous solution of Congo-red, and allowed to dry. It is then cut into narrow strips. A piece of the paper is moistened with the stomach contents. Free hydrochloric acid turns the paper deep blue; lactic acid produces a much less intense blue. The test is fairly delicate, but with very dilute solutions of hydrochloric acid the color change is very slight and rather difficult to interpret. Lactic acid is never found in sufficient concentration to lead to difficulty, according to Riegel.

5. **Töpfer's Test.**—One drop of 0.5 per cent alcoholic solution of dimethylamidoazobenzol is added to a few c.c. of gastric juice. Free hydrochloric acid produces a bright red color. Organic acids also cause a color reaction, but the color is less brilliant—more of a brick red. The reaction is, therefore, not specific, and is the least reliable of the tests.

Of the tests for free hydrochloric acid, *the Günzberg test is the most delicate and at the same time the most reliable.* It is a good routine test, and in any case should be employed wherever doubt exists.

In certain instances where it is desirable to have information regarding the acid secretion of the stomach, contraindications to the passage of the stomach tube exist. In such case Sahli's desmoid test may be used.

6. **Sahli's Desmoid Test.**[4]—This is a test for free hydrochloric acid. It is based on the fact that raw catgut is soluble in hydrochloric acid-pepsin, insoluble in pancreatic and intestinal juices.

Pills of the following formula are prepared:

Methylene blue	0.05 gm.
Iodoform	0.1 gm.
Ext. glycyrrhiz.	q. s.

The pills should not exceed 3 or 4 mm. in diameter. The iodoform may be omitted. The pill is placed in the center of a square of thin rubber dam, such as dentists use. The rubber is stretched and twisted about the pill. The twisted neck is then tied with three turns of *raw* No. 00 catgut, previously soaked in cold water till soft. Now trim the rubber so that a free edge of about 3 mm. width remains beyond the ligature. The cut edges of the rubber must not cohere, inclosing air, for the pill must sink in water, and it must be watertight.

A pill prepared as described is given to the patient with his midday meal, and the urine, collected 5, 7, 18, and 20 hours afterward, is examined for the presence of methylene blue, iodin, or both. In the absence of the greenish color of methylene blue, the urine should be boiled with one-fifth volume of glacial acetic acid. If the chromogen of methylene blue exists in the urine, the color will then appear. Iodin may be looked for with Obermayer's test for indican (p. 24). If methylene blue appears in the urine within twenty hours after the administration of the pill, the test is considered positive.

A positive test shows that there is sufficient free hydrochloric acid secreted in the stomach to permit of digestion of the raw catgut and liberate the pill from its rubber capsule. If the gastric juice fails to digest the catgut, the pill passes into the intestines and is evacuated. The test is, therefore, one for free hydrochloric acid. As it is given with a regular meal, it encounters the optimal conditions for acid secre-

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[4] Boggs, T. R. "Sahli's desmoid reaction in gastric diagnosis." *Bull. Johns Hopkins Hosp.*, 1906, XVII, 313.

tion. The test is thus a useful adjuvant to the usual gastric analyses in certain cases of anacidity.

Organic Acids.—When free hydrochloric acid is markedly diminished or entirely lacking, tests for organic acids should be made. With normal hydrochloric acid values, lactic acid fermentation does not occur. The tests for organic acids are described on pages 132-134.

Quantitative Determination of Gastric Acidity

In the quantitative analysis of the gastric juice the amount of free hydrochloric acid and of total acidity and the extent of the hydrochloric acid deficit are of importance clinically. Very little of diagnostic value has resulted from estimation of the loosely combined hydrochloric acid, that is, hydrochloric acid in protein combination.

The variations in the quantity of free hydrochloric acid are given by Sahli as follows:

Hydrochloric acid is normal:

1. In *health.*
2. In *atony* of the stomach.
3. In *gastric neuroses* at times.
4. In *gastric ulcer* at times.

Hyperchlorhydria (*hyperacidity*) *may be found:*

1. In *gastric ulcer,* usually.
2. In *hypersecretion.*
3. In *gastric neuroses* at times.
4. In *chlorosis,* usually.
5. In the early stages (irritative) of chronic *gastric catarrh.*
6. In many *mental disorders.*
7. During the *menstrual period* at times (Wolpe).

Hypochlorhydria (*subacidity*) *may be found:*

1. In *fevers.*
2. In severe *anemias,* especially secondary anemias.
3. In *gastric neuroses* at times.
4. In many *mental diseases.*
5. In cases of *jaundice* of long duration.
6. In chronic *gastric catarrh.*

7. In chronic *cachexias,* such as tuberculosis.
8. In chronic *passive congestion.*
9. In chronic *nephritis* at times.
10. In diseases of the *gall-bladder* at times.

Achlorhydria (anacidity) may occur:

1. In all of the *conditions listed under hypochlorhydria,* when of severe grade.
2. In *cancer* of the stomach, usually.
3. In *atrophic gastritis.*
4. In *pernicious anemia.* (Usually, the lack of free hydrochloric acid in this disease is due to atrophy of the gastric mucosa.)
5. In association with *angina pectoris* frequently (personal observation).

Töpfer's Method for Free Hydrochloric Acid.—This is the method generally employed, since it is quickly carried out and is sufficiently accurate for clinical purposes.

A drop of 0.5 per cent alcoholic solution of dimethylamidoazobenzol is added to 10 c.c.[5] of filtered gastric contents, placed in a porcelain dish or in a beaker resting on a sheet of white paper for a background. The gastric juice should be measured accurately with a pipette. In the presence of free hydrochloric acid, the addition of the drop of indicator produces a brilliant red color in the liquid. From a burette graduated in tenths of a cubic centimeter, tenth normal sodium hydrate is run into the mixture, a few drops at a time, with constant stirring, till the red color entirely disappears. This is the end reaction. The quantity of tenth normal hydrate required to neutralize the acid in 10 c.c. of the gastric contents is then read from the burette.

The result is usually expressed as "acidity per cent," that is, the number of cubic centimeters of tenth normal alkali which would be required to neutralize the free acid in 100 c.c. of gastric contents. Since 10 c.c. were taken, the quantity of alkali used, multiplied by 10, gives the desired result. *Normally,* free hydrochloric acid varies between 20 and 40. The amount of hydrochloric acid may be calculated. One c.c. of tenth normal alkali is equivalent to 0.00365 gm. HCl.

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[5] If the quantity of gastric contents obtained is small the titration is made with 5 c.c., with a corresponding correction in the final calculation.

If the amount of gastric juice is small, the same sample may be employed for the determination of total acidity. A drop of phenolphthalein is added and the titration continued. The alkali used in neutralizing the free hydrochloric acid must, of course, be included in the total acidity.

Dimethylamidoazobenzol is not the ideal indicator, since it reacts with organic acids and acid salts as well as with mineral acids. The results obtained with it are, therefore, too high; they do not represent absolute values. Nevertheless, the method fulfills all clinical needs, since the error introduced is relatively so small that it does not vitiate the results for diagnostic purposes.

Other Indicators.—In place of dimethylamidoazobenzol Günzberg's reagent and Congo-red are frequently employed as indicators in the titration of free hydrochloric acid.

Günzberg's reagent may be used in several ways. As the titration progresses, a small drop of the gastric juice is removed with the stirring rod from time to time, and placed on the evaporated Günzberg's reagent. The drop is evaporated, and the red color appears at the margin as long as free acid exists. A second procedure consists in the addition of 25 to 30 drops of Günzberg's reagent to the gastric juice, and then at intervals the removal of a minute drop, which is evaporated in the usual manner. The glass stirring rod itself may be gently warmed till the fluid clinging to it is evaporated; it is then examined for the red color. The disadvantage in these procedures is that a small quantity of the gastric contents is lost with each test for free acid, so that the result is slightly low. Comparative titrations with Günzberg's reagent and dimethylamidoazobenzol will show less free acid, as a rule, when Günzberg's reagent is used; occasionally the values are alike.

Congo-red paper may also serve as the indicator in the titration of free hydrochloric acid. It is very convenient for night work. The tenth normal alkali is added to the gastric juice until a small drop placed on Congo-red paper no longer produces a blue color. As a control the paper should be moistened with distilled water, for the red color becomes somewhat darker when moistened. The results are usually intermediate between those obtained with Günzberg's reagent and those with dimethylamidoazobenzol.

Titration of Total Acidity.—The total acidity comprises free hydrochloric acid, loosely combined hydrochloric acid (that is, in combination with protein), acid salts, and organic acids, such as lactic, butyric, and

amino-acids, when present. Its quantity is determined by titration with tenth normal alkali, using phenolphthalein as the indicator.

Total acidity of the gastric contents usually parallels the free hydrochloric acid fairly closely. However, in cases of hyperchlorhydria, there may be a disproportionate increase in total acidity; in achlorhydria associated with cancer (also in other instances of achlorhydria, where stagnation of the gastric contents occurs), there may be a high total acidity, due largely to the presence of organic acids.

Method.—With a pipette measure 10 c.c. (or 5 c.c.) of filtered gastric contents into a porcelain dish or Erlenmeyer flask placed on a sheet of white paper, and add one or two drops of 0.5 per cent alcoholic solution of phenolphthalein as indicator. In an acid medium it is colorless, but it becomes pink as soon as all the acid is neutralized, leaving a slight excess of alkali. Tenth normal sodium hydrate is added from a burette under constant stirring, until the whole mixture takes on a faint pink color, which persists. The number of c.c. of alkali used, multiplied by 10 (or by 20 in case 5 c.c. of gastric contents were taken), gives the total acidity per cent. *Normally,* this varies between 40 and 60 or 70.

The results obtained are again only approximately correct, being too high as a rule. For diagnostic purposes the method is practicable.

The Hydrochloric Acid Deficit

A deficit in hydrochloric acid occurs whenever the gastric mucosa secretes so small a quantity of hydrochloric acid that there is not merely an absence of free acid, but an excess of bodies capable of binding or uniting with it. Such bodies are chiefly proteins and their end-products, peptids, and the amino-acids. If peptic digestion of the proteins alone occurs, the amino-acids are not concerned in the production of a deficit in hydrochloric acid, since pepsin is unable to carry the hydrolysis of the protein molecule to the amino-acid stage. But, when trypsin is regurgitated into the stomach, or when the proteolytic enzyme of a malignant neoplasm is secreted into the stomach, amino-acids may be abundant in the stomach contents; they may also be the result of bacterial decomposition, though probably not frequently. The presence of amino-acids is of significance in two directions in the quantitative analysis of the gastric contents, as Fischer [6] has pointed out. Pepsin converts the pro-

[6] Fischer, H. "Zur Kenntniss des carcinomatösen Mageninhaltes." *Deutsch. Archiv f. klin. Med.,* 1908, XCIII, 98.

teins into peptids, which react alkaline toward litmus; when united with hydrochloric acid, the reaction is reversed. The hydrolysis of the peptids into their constituent amino-acids alters the conditions. The latter can bind hydrochloric acid, and at the same time carboxyl groups are liberated. The result is that the total acidity is increased, while the free hydrochloric diminishes. With an excess of amino-acids it is then necessary to add more or less hydrochloric acid before a reaction for free acid is obtained. Factors which play a less important rôle in the production of an acid deficit are alkalies introduced with the food or secreted, possibly, in disease.

Hydrochloric acid deficit has been found:

1. In *cancer* of the stomach.
2. It may be encountered, usually of less degree, in any condition causing *achlorhydria.*

It is unnecessary to remark that only those specimens of gastric juice which fail to react to Günzberg's reagent for free hydrochloric acid are suitable for the determination of a deficit in acid.

The *method* of determining the deficit in free hydrochloric acid is as follows: From a burette add tenth normal hydrochloric acid to 5 or 10 c.c. of the gastric contents with constant stirring, until a test for free hydrochloric acid is obtained. For this purpose the Günzberg test is to be preferred. Dimethylamidoazobenzol is not well adapted to the titration, since organic acids which are often present react with it; Congo-red paper gives more satisfactory results than dimethylamidoazobenzol. The extent of the deficit may be expressed as "deficit per cent," —the usual way; the number of cubic centimeters of tenth normal hydrochloric acid which would be required for 100 c.c. of gastric contents is calculated. Or the deficit may be expressed in terms of hydrochloric acid, calculated for 100 c.c. of stomach contents.

Organic Acids

Lactic Acid

Of the organic acids which may be present in the stomach contents in disease, lactic acid is the most important and is the only one tested for in the usual routine examination. It is odorless. Lactic acid is the result of fermentation of the gastric contents. The fermentation occurs only in the absence or very marked decrease of free hydrochloric

acid. When many Oppler-Boas bacilli are present in the gastric contents, lactic acid is usually found, though the converse is not true. Lactic acid almost always means stasis of the gastric contents; it is not found in anacidity, where the motor power of the stomach is normal. The combination of anacidity and stasis, so common in cancer of the stomach, accounts for the frequency with which lactic acid is found in this disease. Quantitative estimation of lactic acid has not been found of value in diagnosis.

Qualitative Tests for Lactic Acid.—1. Ueffelmann's Test.—To 15 or 20 c.c. of 1 per cent aqueous carbolic acid in a test tube, 10 per cent ferric chlorid solution is added till an amethyst color is produced; usually 1 to 2 drops suffice. If necessary, the solution is diluted till it is transparent, and is then divided equally between three tubes. To the first a few drops of the filtered gastric contents are added, to the second a like quantity of distilled water to serve as a control, and to the third the same amount of dilute lactic acid solution for comparison with tube one. A yellowish-green (canary yellow) color denotes lactic acid or its salts. A similar color reaction may also be given by oxalic, citric, and tartaric acids, by alcohol and dextrose, but these substances can usually be excluded after an Ewald or Dock breakfast.

To avoid error from disturbing bodies, it has been recommended to extract the gastric contents with about ten volumes of ether, which is then evaporated; the residue is dissolved in water, to which the test is applied.

2. Strauss' Test.—To avoid the sources of error in the preceding test, Strauss employs a specially devised separating funnel, which is used to extract the gastric contents. Above the glass stopcock there are two marks which correspond to 5 c.c. and 25 c.c. The gastric contents are added to the mark 5, and then ether is poured to the mark 25. The two fluids are mixed thoroughly by shaking, and after they have separated the gastric contents are allowed to escape. Distilled water is then added till the ether again rises to the mark 25. After the addition of one drop of 10 per cent ferric chlorid solution, shake vigorously, and wait for the fluids to separate. In the presence of lactic acid a greenish-yellow color is imparted to the watery layer. The extraction with ether separates the lactic acid from the interfering bodies. If lactic acid is combined with protein, the test may be negative; but the lactic acid may be freed by the addition of dilute hydrochloric acid, until a test for the latter is given with Congo paper. The test now becomes positive.

3. Kelling's Test.—A small portion of the gastric contents is diluted with 10 to 20 volumes of distilled water. A second test tube is filled with the same quantity of water alone. To each tube add one drop of 10 per cent ferric chlorid. Lactic acid causes a canary-yellow color. Dilutions of 1:10,000 to 1:15,000 may give a positive reaction. The second tube, containing water and ferric chlorid, serves as a control. As in Uffelmann's test, the color is often perceived most easily by looking down into the test tube, which is held on a white background.

Butyric Acid

Butyric acid fermentation may take place in the presence of considerable quantities of free hydrochloric acid. The odor of butyric acid, resembling that of rancid butter, is characteristic. Boiling the gastric contents accentuates the odor; if a piece of moistened blue litmus paper be held in the mouth of the test tube, the volatile acid reddens it as it escapes during the boiling. Butyric acid also has the peculiar property of separating as a drop of oil on the addition of a small amount of calcium chlorid.

Acetic acid, like butyric acid, may be recognized by its odor if present in sufficient concentration. Acetic acid, after careful neutralization with sodium hydrate, with the formation of sodium acetate, gives a blood-red color on the addition of a drop of ferric chlorid.

Mercury in the Stomach Contents

In cases of mercuric chlorid poisoning, the *gastric contents and feces* should be tested in addition to the urine. The method is the same as that employed for the urine (see p. 73), although the quantity of material taken is usually smaller. The material must be well mixed to insure obtaining a uniform sample of the specimen, especially in the case of the stomach contents if egg albumin has been given as an antidote, as the mercury is then in the form of an albuminate. The oxidation also takes longer, and larger quantities of potassium chlorate are required. Before the wire is placed in the solution, the latter should be filtered in order to remove any fatty substances, carbon, or other insoluble materials.[7]

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[7] Vogel, K. M. and Lee, O. I. "Detection of mercury in the excretions." *Jour. A. M. A.*, 1914, LXII, 532.

GASTRIC FERMENTS

Normally pepsin and rennin, or their zymogens, are constituents of the gastric juice. Alterations in the enzymes in disease are much less frequent and less striking than those occurring in the hydrochloric acid. Whenever the latter is present, it is practically always the case that pepsin is also found. With absence of free hydrochloric acid, tests for the enzymes should be made. Quantitative determination of pepsin has not proved to be sufficiently valuable to warrant its inclusion in the usual routine gastric examinations.

Pepsin

Qualitative Test for Pepsin.—Discs of coagulated egg albumin, ca. 1.5 mm. thick and 5 to 10 mm. in diameter, are cut with a cork-borer or goose-quill. They may be preserved in glycerin, but should be washed in water immediately before use to remove the excess of glycerin. A disc of the coagulated albumin is placed in a few c.c. of gastric contents, and, if necessary, dilute hydrochloric acid is added, till Congo paper gives a test for free acid. The material is then placed in an incubator at 37° C. (or in the vest pocket). In one-half to one hour the albumin should be digested.

Fibrin, usually obtained from ox blood and preserved in glycerin, may be substituted for the coagulated egg albumin.

Quantitative Methods.—Quantitative methods for pepsin may occasionally be desirable. Several have been proposed within the last few years. The results given by each are relative, not absolute, values.

Mette's Method as Modified by Nierenstein and Schiff.[8]—Capillary glass tubes, 1 to 2 mm. in diameter and 20 to 30 cm. in length, are filled with egg albumin by suction, the ends plugged with bread crumbs, and the tubes then placed in boiling water for five minutes. They are then sealed with paraffin or sealing wax. Bubbles appear in the albumin, but are no longer seen after three days, when the tubes are ready for use. If the albumin retracts from the wall of the tube, it should not be used for the test.

Method.—One c.c. of filtered gastric contents is diluted with 15 c.c. of twentieth normal hydrochloric acid. With a file or glass scissors,

[8] Farr, C. B., and Goodman, E. H. "The clinical value of the quantitative estimation of pepsin, with special reference to the Mette and ricin methods." *Arch. Int. Med.*, 1908, I, 648.

cut off about 2 cm. of the capillary tube, and place two such pieces in the diluted gastric contents. The test tube is then corked and placed in an incubator at 37° C. for twenty-four hours. At the end of this time the tubes are removed and the amount of digestion of albumin in the four ends of the capillary tubes is measured in tenths of a millimeter, a hand lens being useful for this purpose. An average of the four readings is taken. The square of this number represents the number of units of pepsin present in the diluted gastric contents. Multiplying this by 16 gives the value for the undiluted specimen.

Since the albumin from different eggs may react differently, and since the length of time the albumin is boiled affects its digestibility, the method can be relied upon only to show rather wide variations in pepsin.

According to Cowie,[9] the tubes need not remain in the incubator twenty-four hours. He finds that the amount of digestion varies directly as the time. He derives the following formula for calculating the digestion:

If A=the amount of egg white digested,
B=the time the tubes remain in the incubator,
C=the required time for the end reaction,
X=the peptic value of the fluid tested, or the estimated value in millimeters,

then it will be found that $X=\frac{A \times C}{B}$

Rennin

Rennin, the enzyme responsible for the coagulation of milk, may exert its characteristic action in the absence of hydrochloric acid. Rennin zymogen, inactive in itself, is converted into rennin by acid. The zymogen is much more resistant to alkalies than rennin.

Qualitative Test for Rennin.—Three to 5 drops of filtered gastric contents are added to 5 to 10 c.c. of raw, amphoteric, or neutral milk. After mixing thoroughly, the fluid is placed in the incubator for 15 to 20 minutes. The presence of rennin is shown by the curdling of the

[9] Cowie, D. M. "A rapid procedure for the estimation of the peptic value of stomach fluid by means of the Mette method." *The Phys. & Surg.*, Detroit and Ann Arbor, 1904, XXIV, 118.

milk, provided its reaction remains neutral or amphoteric. If the reaction has become acid, it is probable that fermentation or "souring" of the milk is the cause of the curdling. Riegel recommends that equal parts of milk and gastric contents be taken. The latter is first neutralized with tenth normal alkali. Curdling should occur within 15 to 30 minutes. Acid reaction of the milk after incubation invalidates the test, as in the preceding instance.

Rennin Zymogen

Riegel gives the following method for detecting rennin zymogen: Ten c.c. of the gastric contents are rendered alkaline with tenth normal sodium hydrate to inactivate rennin. Then add 10 c.c. of fresh, neutral, or amphoteric milk and 3 to 5 c.c. of 1 to 2 per cent calcium chloride solution, and place the mixture in the incubator at body temperature. If the zymogen is present, casein is precipitated within a few minutes.

MUCUS

Mucus is always present in the gastric juice, though in very small amount under normal conditions. When present in excess, the characteristic ropy or stringy quality of the fluid is seen on pouring it from one vessel to another. Microscopically, small snail-like masses of mucus may be seen. Mucus usually contains epithelial or pus cells, if the latter are present in the stomach contents; with normal acidity digestion may leave only the nuclei. Mucus from the bronchi, pharynx, or esophagus is often observed in the gastric contents. Bronchial mucus usually contains bubbles, which cause it to float. Microscopic examination reveals alveolar cells, often laden with coal dust, and an abundance of pus cells frequently; an absence of food particles is often noted. The mucus from esophagus and pharynx, which is generally formed in abundance during the passage of the tube, runs out along the side of the tube. It should not be allowed to mix with the material obtained from the stomach.

Anacidity often causes an apparent increase in mucus, even though there is no overproduction, since that which is formed is not normally digested, and, furthermore, it swells to an unusual degree. Mucus is increased in the early (irritative) stage of gastric catarrh; in fact, in any irritative lesion of the stomach associated with subacidity or anacidity and without atrophy of the mucosa (that is, the cells secreting mucus),

an excess of mucus may be encountered. It is seen at times in gastric neuroses.

MICROSCOPIC EXAMINATION OF THE GASTRIC CONTENTS

Normally one finds, after a test breakfast, only isolated bacteria, a few desquamated epithelial cells, many starch granules, a few fat droplets, possibly a few yeast cells (not budding), an occasional leukocyte at times, and small particles of mucus. The fresh specimen should be employed for examination, which is made with the dry objectives.

Starch granules (Fig. 22) are conspicuous. When well preserved, laminations are indicated by the concentric lines. If digestion has not proceeded too far, the starch granules are stained deep blue on the addition of a drop of Lugol's iodin solution.

Fat droplets are recognized by their appearance and staining reactions (see p. 77).

Blood corpuscles, when present in the gastric juice, are often too greatly damaged to be recognized microscopically. With a recent hemorrhage they may, however, present a characteristic appearance, particularly if enough blood has been shed to completely bind the free acid.

Blood may be found in the gastric contents in association with:

1. *Gastric ulcer,* at times also in *duodenal ulcer* from regurgitation of the duodenal contents.
2. *Cirrhosis of the liver* or *splenomegaly* with *esophageal varices.*
3. *Cancer* of the stomach.
4. Through *swallowing of blood* from the nose, throat, mouth, etc.
5. *Traumatism* from passing the stomach tube (traces).
6. Rarely from *leakage of an aneurysm* into the esophagus.
7. The *malignant forms* of the *acute infectious diseases.*
8. In certain cases of *purpura.*
9. In severe *anemias* and in *acute leukemias* at times.
10. *Vicarious menstruation* (mentioned by Hippocrates).
11. After *laparotomies* involving the upper part of the abdomen.
12. From *trauma.*
13. *Hemorrhagic erosions,* after long continued vomiting.
14. The *erythema group of skin diseases* at times.

Unless the blood is bright red or clotted, chemical tests may be required for its recognition. It must be remembered, too, that the blood of a

gastric hemorrhage may be evacuated entirely in the feces, which should be tested chemically for blood in all cases of disease of the stomach.

A negative test for minute quantities of blood in the gastric contents ("occult" blood) excludes swallowed blood or blood from trauma in the passage of the tube. With a positive test, the examiner must exclude both of these possible sources of error.

The guaiac or benzidin tests are usually employed as preliminary tests. (For the technic of the *chemical tests* see the section on the feces pp. 147-149.)

Pus cells (Fig. 22), when well preserved, differ in no way from

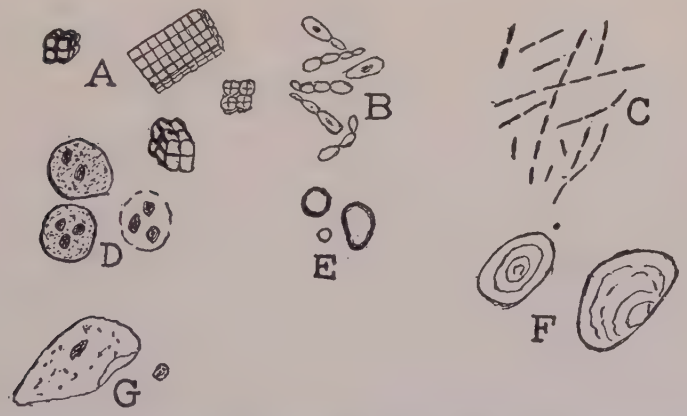

FIG. 22.—GASTRIC CONTENTS. *A*, sarcines; *B*, yeasts; *C*, Oppler-Boas bacilli; *D*, pus cells, intact, and digested, except nucleus; *E*, fat droplets; *F*, starch; *G*, epithelial cell.

those observed elsewhere. Generally the protoplasm of the cells has been digested, leaving only the naked polymorphous nuclei.

Eosinophilic leukocytes are a rare finding in the gastric contents.[10]

Micro-organisms may exist in the stomach in large numbers in disease. Normally their growth is prevented by the free hydrochloric acid and the rapid emptying of the organ.

Yeasts (Fig. 22) are introduced into the stomach in small number with the food, but they exhibit no sign of germination. In disease, on the other hand, often in the presence of considerable free hydrochloric

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[10] Moačanin, S. "Ueber das Vorkommen von eosinophilen Zellen im Magensafte bei Achylia gastrica." *Wiener klin. Wchnschr.*, 1911, XXIV, 1335.

acid, an active growth of yeasts may be found. Large colonies may be observed. The cells are oval bodies, smaller than a red corpuscle, which have a greenish, glistening appearance when seen with strong illumination. Characteristic budding forms—three or four cells linked together, with a progressive diminution in size—are common. They may be distinguished with the low power, and are easily recognized with the high power, dry objectives.

Sarcinæ (Fig. 22) are found in the form of bales or packages, or as irregular masses of cells. Like yeasts, they are abundant in the stomach only in disease. Two sizes, large and small, are met with, and the significance of each is the same. They are slightly brownish, and can often be found most easily with the low power. They are usually associated with stasis of benign origin.

Oppler-Boas bacilli (Fig. 22) are capable of producing lactic acid fermentation, and thus it happens that their growth practically always results in the simultaneous presence of lactic acid in the stomach contents. The bacilli are characterized by their great size and lack of motility. They are long and have a tendency to grow in chains, which may at times extend across the field of the microscope. To be of importance, the organism must be present in large numbers. It is Gram-positive. It is seen without difficulty with high power, dry objectives in the fresh, unstained preparation. The bacilli are more frequently associated with malignant disease than with other conditions in the stomach.

Trichomonas intestinalis[11] (see p. 168, Fig. 25), is rarely found in the stomach. Other protozoa—*Balantidium coli, Giardia intestinalis*—are very uncommon. (For descriptions of these parasites see pp. 168-169, and 171.) Protozoa are found only in the absence of free hydrochloric acid. In most cases, when present, they are associated with carcinoma of the stomach, especially in cases in which the cancer is ulcerating (Cohnheim).

Crystals are of little importance in the stomach. In bile-tinged specimens cholesterin crystals and spheres of leucin have been observed. Triple phosphate, fatty acid, and oxalic acid crystals have been noted.

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[11] Cohnheim, P. "Infusorien bei gut- und bösartigen Magenleiden nebst Bemerkungen ueber die sogenannte Infusorienenteritis." *Deutsche med. Wchnschr.*, 1909, XXXV, 92.

CHAPTER III

THE FECES [1]

In the examination of the feces it is a matter of prime importance that the material be obtained as fresh as possible. If the examination is delayed beyond a few hours at the most, it is quite possible, and, in fact, probable, that erroneous conclusions will be reached. This is particularly the case with regard to animal parasites. It is necessary, for example, to examine for amebæ before the material has cooled; hookworm ova may hatch in the stool, under favorable conditions, in twenty-four hours. Furthermore, bacterial digestion of food rests, such as muscle fibers, may proceed to such a degree within a comparatively short time after the stool has been passed as to lead to a false impression on microscopic inspection. Were it necessary, examples might be multiplied almost indefinitely.

MACROSCOPIC EXAMINATION OF THE FECES

The approximate amount, form, consistence, and color of the stool are noted, and also all recognizable pathological elements, such as parasites, mucus, blood, pus, gall-stones, undigested portions of food, etc. A simple inspection suffices for the determination of most of these points.

Amount.—The amount of the feces in health varies between about 120 and 250 gm. in twenty-four hours. The frequency of defecation and the quantity of food eaten largely determine the amount passed at any one time.

Form.—The formed or soft stool of the normal individual requires no description. *Scybala* are the small, hard masses of fecal material which have remained in the bowel too long and have become abnormally

[1] As reference works in the study of the feces wide recognition has been accorded *Makro- und mikroskopische Diagnostik der menschlichen Exkrement* by M. L. Q. van Ledden Hulsebosch, Berlin, 1899, and to *Die Faezes des Menschen* by Ad. Schmidt and J. Strasburger. Berlin, 1910, 3d. ed. Both are profusely illustrated.

dry. They are at times coated with mucus, and not infrequently fresh blood may be seen on their surface. The size of a formed stool should be noted; the small movements, about the thickness of a lead pencil, which are seen in starvation or in pathological states of the large intestine, are abnormal. In diarrhea the stools are fluid.

Color.—The color of the stools *in health* is derived largely from (1) *hydrobilirubin* (*urobilin*), which is reduced bilirubin; though in breast-fed infants' stools unaltered bile pigment is met with. (2) *Food* may alter the color of the intestinal discharges. With a milk diet, the color is light. An unusually dark color results from eating blueberries, etc., and from drinking red wines. Vegetables rich in chlorophyll, such as spinach, may impart a dark green or olive tint. (3) Certain *drugs* have a marked effect on the color of the intestinal contents. After *calomel* a greenish color may be noted; *bismuth* salts may cause a dark brown or even a black color, due to the black crystals of bismuth suboxid. A normally pigmented stool, which becomes dark on exposure to the air, is often attributable to the use of *iron*. Similarly, if *methylene blue* be administered by mouth, oxidation after the stool has been passed may lead to a dark bluish-green color on its surface.

Among the *abnormal coloring matters* of the feces, (4) *blood* is of great importance. The extent to which the color is altered depends upon the size of the hemorrhage and its source—whether high or low in the gastrointestinal tract. Very small hemorrhages in the stomach or small intestine produce no perceptible change in the appearance of the feces; these are the so-called "occult hemorrhages," which are recognized only by chemical tests. On the other hand, large gastric or duodenal hemorrhages lead to the so-called "tarry" or black stools. A hemorrhage of any considerable size low in the ileum often manifests itself by the passage of very dark red clots or fluid, the hemoglobin showing less alteration than in the preceding instance. With rectal hemorrhages the blood is bright red, often unclotted, and is seen on the surface of the stool, not intimately mixed with it unless the stool is a fluid one. (5) *"Clay-colored"* stools gain their name from the resemblance to white clay. They may be due to (*a*) entire absence of bile pigment, *acholic stools,* with the usual increase of fat which accompanies this condition; (*b*) the reduction of bilirubin by bacteria may be excessive, giving rise to a colorless compound, *leukohydrobilirubin.* In this case the surface of the stool becomes dark after more or less prolonged exposure to the air, and, unlike acholic stools, hydrobilirubin

is demonstrable; (*c*) with very *excessive fat* content, the normal fecal pigment may be so greatly obscured that the stool is clay-colored. (6) The presence of very large quantities of *pus* or of fluid may cover or dilute the normal pigment to such an extent that the specimen appears lighter than usual. Pure pus, such as one sees after the rupture of an abscess into the rectum, needs no description.

Mucus.—Mucus is present in normal feces, but not in sufficient quantity to be observed macroscopically. Long strings or ribbon-like masses of mucus, tenacious and slimy, usually slightly stained with urobilin, may be observed in disease, or, again, the stools may be encased in mucus, giving an appearance very suggestive of a membrane or a sausage-skin. More frequently smaller particles of mucus are found, varying in size from a split pea up to that of an almond or larger, at times blood-stained or mixed with pus or eosinophilic cells.

Gall-stones.—Gall-stones or other concretions may be found, when present, in the following manner: A bowl or other vessel of about one liter capacity is lined with a double thickness of surgical gauze of sufficient size to permit the free margin to extend well beyond the edge of the bowl on all sides. The stool is now placed on the gauze in the bowl, and the free edges of the gauze are securely tied, so that the stool is contained in a bag of gauze. The specimen is left in the bowl, which is now placed under a stream of running water, where it is allowed to remain until all the finer particles of the feces have been washed away. Gall-stones, unless very minute, cannot pass through the meshes of the gauze, and are, therefore, found in the bag.

This procedure is applicable to the detection of the larger fruit seeds, foreign bodies, etc.

Parasites.—Parasites, such as Ascaris lumbricoides and the larger cestodes, are striking objects which arrest attention at once. Methods for the detection of the smaller worms are described in connection with the hookworm.

INTESTINAL TEST DIET

In the study of functional and anatomic alterations of the intestine, a uniform diet is desirable for many reasons; microscopic and chemical examinations are greatly simplified, and there is supplied a basis of comparison which is not possible when patients are free to choose their own food.

The test diet of Schmidt and Strasburger[2] is that generally used. Five small meals are given, the first on waking, the fourth in the afternoon.

Diet No. I.—*In the Morning.*—One-half liter of milk, or of tea, or cocoa cooked with milk or water. One roll with butter and 1 soft-boiled egg.

Breakfast.—One dish of oatmeal cooked with milk and strained, with salt or sugar as desired. Instead of oatmeal, gruel or porridge may be taken.

Noon.—One-quarter pound of chopped, lean beef, broiled in butter, rare. A fairly liberal portion of potato purée.

Afternoon.—Same as in the morning, without the egg.

Evening.—One-half liter of milk or 1 dish of oatmeal prepared as for breakfast. One roll with butter. One or 2 eggs, soft-boiled or scrambled.

Diet No. II.—For quantitative studies Schmidt and Strasburger[3] recommended a diet containing the following foods, which must be carefully measured: 1.5 liters of milk, 100 gm. of zwieback, 2 eggs, 50 gm. of butter, 125 gm. of beef, 190 gm. of potatoes, oatmeal made from 80 gm. of dry meal, 2 to 3 gm. of salt. They suggest the following arrangement for giving the food:

In the Morning.—0.5 liter milk and 50 gm. zwieback.

In the Forenoon.—Strained oatmeal prepared from 40 gm. of oatmeal, 10 gm. of butter, 200 c.c. of milk, 300 c.c. of water, 1 egg, and salt.

Noon.—125 gm. of chopped beef (raw weight) broiled with 20 gm. of butter; the beef should remain raw on the inside; 250 gm. of potato purée prepared from 190 gm. of mashed potato, 100 c.c. of milk, 10 gm. of butter, and salt.

Afternoon.—Same as in the morning.

Evening.—Same as in the forenoon.

The authors usually give the diet for three days, occasionally longer. To determine when the food has passed through the intestinal tract, they give 0.3 gm. of powdered carmine in capsule with the first meal of the test diet. The carmine produces a red color in the feces.

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[2] Schmidt, A., and Strasburger, J. *Loc. cit.*, pp. 5-6. *Also* Schmidt, A. *The examination of the function of the intestines by means of the test diet,* etc. (Translated by C. D. Aaron.) Philadelphia, 1909.

[3] *Loc. cit.*

This second diet, it is calculated, contains 102 gm. of protein, 111 gm. of fat, and 191 gm. of carbohydrate. It is equivalent to 2,234 calories.

Weight of Dried Feces.—The feces are dried on a steam or water bath. The weight of the dried feces of normal adults on Schmidt's diet No. II varies between about 45 and 62 gm.

CHEMICAL EXAMINATION OF THE FECES

For chemical examination a fresh specimen of feces should always be used.

Reaction.—The reaction of normal feces is neutral, faintly alkaline, or faintly acid. It is tested with litmus paper. If the stool is formed or soft, a small portion for testing should be rubbed in a mortar with a little distilled water.

Pigments.—The normal fecal pigment is hydrobilirubin (urobilin). In breast-fed children, however, the bilirubin is not reduced by bacteria, and appears as such in the feces. In disease unaltered bilirubin may be present in the feces; the same is true after active purgation.

Urobilin (*Hydrobilirubin*)

1. **Schmidt's Test.**—A portion of the fresh feces the size of a hazelnut or larger is rubbed in a mortar with three to four times its volume of concentrated watery solution of bichlorid of mercury. The suspension obtained is placed in a covered Petri dish, and set aside for twenty-four hours. All particles stained with hydrobilirubin (urobilin) are colored red, whereas bilirubin becomes green. The color change may appear in less than an hour. The material may be examined microscopically, when even minute particles which contained bilirubin become evident by their green color.

2. **Schlesinger's Test.**[4]—A portion of the stool is rubbed in a mortar with distilled water to obtain a thin watery suspension. If much fat is present, extract the suspension with ether twice to remove it. Then treat the suspension with acid alcohol (HCl 3 c.c., alcohol to 100 c.c.), and later neutralize the acid with ammonia. Now add to the mixture an equal volume of saturated alcoholic solution of zinc acetate, mix thoroughly, and filter. In the presence of urobilin a green fluorescence is seen.

[4] Schlesinger, W. *Loc. cit.* (p. 64).

3. **Spectroscopic Determination.**—The watery suspension of feces is acidulated with acetic acid, and is then extracted with amyl alcohol. The extract is examined for the bands of urobilin (see p. 64).

Bilirubin

1. **Schmidt's Test.**—This is applied as just described for urobilin. A green color denotes the presence of bilirubin. It permits the recognition of even microscopic bilirubin-stained particles in the presence of an excess of urobilin.

2. **Gmelin's Test.**—This test is applicable only when a great excess of bilirubin is present (Schmidt and Strasburger). The feces must be examined while fresh. A watery suspension of the material is prepared. Filter paper is soaked in the suspension, and then a drop of yellow nitric acid is placed on the paper. The characteristic play of colors is seen about the edge of the drop—yellow, red, violet, blue, and green, the last at the periphery.

Blood

Blood may be demonstrated *chemically* in the feces *in any of the conditions enumerated under blood in the gastric contents* (p. 138; see also p. 142). The patient should be placed on a diet free from hemoglobin and chlorophyll (green vegetables) for two days before the tests are made, to exclude error from this source. It is manifest that bleeding may occur at any point below the stomach. Thus, blood may be found in the stools as the result of:

1. *Duodenal ulcer.*
2. *Typhoid ulcers.*
3. *Tuberculous ulcers.*
4. *Acute enterocolitis* frequently.
5. *Dysentery,* both bacillary and amoebic.
6. *Intestinal parasites,* especially *Schistosoma mansoni;* with *hookworm* disease, "occult" hemorrhage is not uncommon.
7. *Hemorrhoids.*
8. *Malignant neoplasms.*
9. In *malignant fevers, hemorrhagic diseases* (purpuras), etc.
10. Following injections of *arsphenamine* (rare).

1. **Weber's Test.**—A watery suspension of feces is prepared in a mortar. If the stool contains much fat, this is removed by extraction

with ether. Then add to the suspension one-third volume of glacial acetic acid and mix thoroughly. If blood is present, the coloring matter is converted into acid hematin. The mixture is now filtered and the filtrate extracted with two to three volumes of ether. Separation of the ether may be hastened by the addition of a few drops of alcohol. Depending upon the quantity of blood present, the ether extract shows a more or less intense shade of brown. The extract is now examined spectroscopically for the bands of acid hematin (see p. 63).

2. **The Guaiac Test.**—If the stool contains too little blood to give the spectroscopic test, about 2 c.c. of the ether extract obtained in Weber's test is treated with about 10 drops of freshly prepared tincture of guaiac (a knife-point of powdered guaiac dissolved in about 5 c.c. of alcohol), and 20 to 30 drops of hydrogen peroxid or old, ozonized turpentine. The mixture is shaken, and in the presence of blood a blue color develops throughout the mixture. The color fades after standing a few minutes.

Cowie [5] reports a "water modification" which he finds more delicate. All glassware should be chemically clean and dry. One gram of feces which has been softened with as little water as possible is rubbed in a mortar with 4 to 5 c.c. of glacial acetic acid. To the suspension obtained add 30 c.c. of ether, and shake. To 1 or 2 c.c. of the ether extract add an equal amount of distilled water, and shake thoroughly. Now a knife-point of powdered guaiac is placed in the test tube, and is dissolved by agitating the contents. Finally 30 drops of old, water-white, pure turpentine (or hydrogen peroxid) are added, and the contents of the tube mixed. The tube is examined against a white background for the color reaction. If blood is present to the extent of 1 mg. in 1 gm. of feces, a distinct light blue color develops quickly in the ether. With larger amounts of blood the color is, of course, more intense.

Sources of Error.—The guaiac test is not a reliable test for blood. It is, however, a very delicate test, and, when it is negative, blood in appreciable quantity is absent. A positive reaction may be given by a great many substances. Of those most apt to lead to difficulty in fecal examinations raw meat, chlorophyll, pus, and salts of the heavy metals are familiar examples. It is advisable to exclude meat and green vegetables from the diet for at least three days before collecting the specimen for examination. All drugs which might interfere with the

[5] Cowie, D. M. "A comparative study of the occult blood tests; a new modification of the guaiac reaction; its value in legal medicine." *Amer. Jour. Med. Sci.*, 1907, CXXXIII, 408.

test, such as preparations of iron, should also be discontinued. A full list of the substances reacting with guaiac and similar substances—phenolphthalein, aloin, benzidin—is given by Kastle.

The value of the test is concisely stated by Kastle,[6] who says: "The general consensus of opinion among those who have given this subject their attention would seem to be that the guaiacum test for blood and similar color reactions are valuable, especially if they lead to negative results, as proving beyond the peradventure of a doubt that blood is absent. On the other hand, if a positive test is obtained, care should be taken to exclude oxidases or peroxidases by boiling, and the salts of the heavy metals and other oxidizing agents by chemical methods, and, if possible, to subject the material under investigation to confirmative tests for blood before finally concluding that blood is present."

3. **Phenolphthalein Test for Blood.**[7]—This test, as modified by Boas, is more delicate than the guaiac test, though subject to the same sources of error. Its chief value is a negative one—when the reaction is negative, it can be concluded that there is no blood in the stool.

The reagent is prepared as follows: 25 gm. potassium hydrate are dissolved in 100 c.c. of distilled water. Phenolphthalein 6 gm. is added and the mixture is shaken vigorously. After solution is complete, powdered metallic zinc is added, and the preparation is boiled in an Erlenmeyer flask until the fluid is completely decolorized—usually two to three hours. After cooling, distilled water is added to the original volume, and the solution is filtered. A water clear solution is obtained, which remains colorless even after adding acetic acid, alcohol and hydrogen peroxid. In the course of a few weeks, oxidation of the phenolphthalein around the edge of the bottle may take place, coloring the first few drops of reagent poured from the bottle. It is always advisable to use a colorless reagent.

The method of performing the test is as follows: An acetic acid-alcohol extract (5 drops of glacial acetic acid and 15 to 20 c.c. of alcohol) of the feces is prepared by suspending a small amount of feces and then filtering. From a dropping bottle, 15 drops of the reagent are placed in a test tube, to which are added 5 to 6 drops of hydrogen peroxid and 2 c.c. of absolute alcohol. The tube is well shaken. The

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[6] Kastle, J. H. "Chemical tests for blood." *Bull. No. 51*, Hyg. Lab., U. S. Pub. Health & Mar. Hosp. Serv., Wash., 1909, pp 1-62.

[7] Boas, I. "Eine Verbesserung der Phenolphthalinreaktion zum Nachweis okkulter Blutungen in den Faeces." *Deutsche med. Wchnschr.*, 1915, XLI, 549.

acetic acid-alcohol extract of the feces is then run into the tube slowly from a funnel, so that the acetic acid-alcohol forms a layer, about one-half of the extract being used.

If blood coloring matter is present, there occurs either immediately or gradually a rose or deep red ring at the line of contact, the depth of color depending on the amount of blood present. If the color is faint, it is more readily perceived by placing the tube before a white background.

4. **Teichmann's Hemin Crystal Test.**—With a minute particle of dried feces, the hemin crystal test may be performed (see p. 73). With very small amounts of blood the test may fail.

Fat and *starch* are usually recognized without difficulty by microscopic examination (see pp. 77, 138, Fig. 22). For quantitative determination of neutral fat and fatty acids, works on chemistry should be consulted. (A new method is described by Folin, O., and Wentworth, A. H. "A new method for the determination of fat and fatty acids in feces," *Jour. Biol. Chem.*, 1910, VII, 421.)

ENZYMES IN THE FECES

The examination of the feces for enzymes of the pancreas has received considerable attention. Trypsin and amylase (diastase) are most often determined, for their relations in the feces are best understood.

Trypsin

Method of Gross [8] for the Determination of Trypsin.

Reagents:

Solution 1:

Caseinum purissimum (Grübler)	0.5 gm.
Sodium carbonate	1.0 gm.
Distilled water	1,000.0 c.c.

Dissolve by very gentle heating, if necessary. Add toluol to prevent bacterial growth.

Solution 2:

Sodium carbonate	1.0 gm.
Distilled water	1,000.0 c.c.

[8] Gross, O. "Zur Funktionsprüfung des Pankreas." *Deutsche med. Wchnschr.*, 1909, XXXV, 706.

The feces to be examined are rubbed in a mortar with three times their bulk of solution 2, until a homogeneous suspension is obtained. This is filtered, till the filtrate is clear. Ordinarily the filtration causes no trouble, but if there is much turbidity from bacteria they settle to the bottom, and the clear fluid may be decanted (Gross). Ten c.c. of the fecal filtrate are placed in a flask with 100 c.c. of the casein solution (solution 1). A few c.c. of toluol are added to prevent bacterial decomposition of the casein. The flask is now placed in the incubator at 37° to 40° C. From time to time small portions are removed and tested for casein; this substance is precipitated by dilute (1 per cent) acetic acid, though the products of its digestion are unaffected. The material is kept in the incubator till the casein has been completely digested. The time is noted.

The rapidity of digestion (and the amount of trypsin) varies with the diet. It is completed most quickly after a protein diet; with carbohydrate food the trypsin is diminished, while intermediate values are obtained with a diet largely of fat. The examination should be made after protein diet; the average time required for complete digestion is 12 to 14 hours, the normal limits being 8 and 15 hours.

Amylase

Wohlgemuth's [9] Method for Determination of Amylase, as Modified by Hawk.[10]—"Weigh accurately about 2 gm. of fresh feces into a mortar (duplicate determinations should be made), add 8 c.c. of a phosphate-chlorid solution (0.1 mol. dihydrogen sodium phosphate and 0.2 mol. disodium hydrogen phosphate per liter of 1 per cent sodium chlorid), 2 c.c. at a time, rubbing the feces mixture to a homogeneous consistency after each addition of the extraction medium. Permit the mixture to stand at room temperature for a half hour with frequent stirring. We now have a neutral fecal suspension. Transfer this to a graduated 15-c.c. centrifuge tube, being sure to wash the mortar and pestle carefully with the phosphate-chlorid solution, and add all washings to the

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[9] Wohlgemuth, J. (a) "Ueber eine neue Methode zur quantitativen Bestimmung des diastatischen Ferments." *Biochem. Ztschr.*, 1908, IX, 1. (b) "Beitrag zur funktioncllen Diagnostik des Pankreas." *Berlin. klin. Wchnschr.*, 1910, XLVII, 92.

[10] Hawk, P. B. "A modification of Wohlegmuth's method for the quantitative study of the activity of the pancreatic function." *Arch. Int. Med.*, 1911, VIII, 552.

suspension in the centrifuge tube. The suspension is now made up to the 15-c.c. mark with the phosphate-chlorid solution and centrifugated for a fifteen-minute period, or longer, if necessary, to secure a satisfactory sedimentation. At this point read and record the height of the sediment column. Remove the supernatant liquid by means of a bent pipette, transfer it to a 50-c.c. volumetric flask, and dilute it to the 50-c.c. mark with the phosphate-chlorid solution. Mix the fecal extract thoroughly and determine its amylolytic activity. For this purpose a series of six graduated tubes is prepared, containing volumes of the extract ranging from 2.5 to 0.078 c.c. Each of the intermediate tubes in this series will thus contain one-half as much fluid as the preceding tube. Now make the contents of each tube 2.5 c.c. by means of the phosphate-chlorid solution in order to secure a uniform electrolyte concentration. Introduce 5 c.c. of a 1 per cent soluble starch solution and three drops of toluol into each tube, thoroughly mix the contents by shaking, close the tubes by means of stoppers, and place them in an incubator at 37° C. for twenty-four hours. (In preparing the 1 per cent starch solution, the weighed starch powder should be dissolved in cold distilled water in a casserole and stirred, until a homogeneous suspension is obtained. The mixture should then be heated with constant stirring until it is clear. This ordinarily takes from eight to ten minutes. A slightly opaque solution is thus obtained, which should be cooled and made up to the proper volume before using.) At the end of this time remove the tubes, fill each to within half an inch of the top with ice water, add one drop of tenth normal iodin solution, thoroughly mix the contents, and examine the tubes carefully with the aid of a strong light. Select the last tube in the series, which shows entire absence of blue color, thus indicating that the starch has been completely transformed into dextrin and sugar, and calculate the amylolytic activity on the basis of this dilution. In case of indecision between two tubes, add an extra drop of the iodin solution and observe them again.

"The amylolytic activity, Df, of a given stool may be expressed in terms of 1 c.c. of sediment obtained by the centrifugation, as above described. For example, if it is found that 0.31 c.c. of the phosphate-chlorid extract of the stool acting at 38° C. for twenty-four hours completely transformed the starch in 5 c.c. of a 1 per cent starch solution, then we would have the following proportion:

$$0.31 : 5 :: 1 \text{ (cc. extract)} : x$$

The value of x in this case is 16.1, which means that 1 c.c. of the fecal extract possesses the power of completely digesting 16.1 c.c. of a 1 per cent starch solution in twenty-four hours at 38° C.

"Inasmuch as stools vary so greatly as to water content, it is essential to an accurate comparison of stools that such comparison be made on the basis of the solid matter. Supposing, for example, that in the above determination we had 6.2 c.c. of sediment. Since the supernatant fluid was removed and made up to 50 c.c. before testing its amylolytic value, it is evident that 1 c.c. of this sediment is equivalent to 8 c.c. extract. Therefore, in order to derive the amylolytic value of 1 c.c. of sediment, we must multiply the value (16.1) as obtained above for the extract by 8. This yields 128.8 and enables us to express the activity as follows:

$$\mathrm{Df}\,\frac{38^\circ}{24\mathrm{h}} = 128.8"$$

This is the method of calculation employed by Wohlgemuth. The departure from the original technic which Hawk has suggested, consists in the addition of the phosphates to the chlorid solution. The object of this is to secure a uniform medium in which the amylase may be examined, for it has been shown that the reaction exerts a marked effect on its activity. To do away with this source of error the phosphate-chlorid mixture is employed.

Wohlgemuth finds that the normal average value is about 150.

MICROSCOPIC EXAMINATION OF THE FECES

Unless the stool be very fluid, it is necessary to dilute it with water before examining it microscopically. The method proposed by Stiles is most satisfactory. A drop of water is placed upon a clean glass slide[11] and then, with a flat wooden toothpick or other suitable instrument, a small quantity of feces is transferred to the drop, and mixed with it. During the mixing the slide is inclined and the mixing is done with an upward stroke. By doing so all gritty, solid particles usually are deposited at the upper end, and do not interfere with the spreading of

[11] Large slides, 2 by 3 in., or small glass plates (photographic plates) are convenient for examining feces, as there is less danger of soiling the hands. The toothpick should, of course, be burned or placed in a disinfecting solution immediately after use. *Feces should always be handled as infectious material,* for it is impossible to know when one is dealing with a typhoid or other carrier.

the specimen under the cover glass, which is applied as soon as a thin, uniform suspension of the feces has been secured. The preparation is now ready for examination. Others prefer to examine the preparation without a cover glass. The specimen is of uneven thickness, and has the further disadvantage that parts of it become dry before the examination can be finished.

The specimen should be searched carefully with the low power, and doubtful objects should be examined with a dry lens of higher magnification. Among the objects to be seen in the microscopic examination are remnants of foods, bacteria, granular débris, cells from the mucosa, from blood, or from exudates, parasites, ova, and crystals.

Food Remnants

Muscle fibers (Fig. 23, *A*) are always seen in the feces of patients on a mixed diet. They are yellow in color. As a result of digestion the ends of the fibers are usually rounded, and often only small particles remain. In some of the fibers, however, the striations are well preserved. When digestion of the fibers is faulty, their number is greatly increased, the striations are preserved in the majority, and the ends are square, not rounded.

Fibrous connective tissue (Fig. 23, *M*) may be observed, particularly when there is a lack of free hydrochloric acid in the stomach, since the fibers, digestible in normal gastric juice, are unaffected by the intestinal and pancreatic secretions.

Curds (Fig. 23, *Q*) may be seen after a rich milk diet and are frequently encountered in infants' stools. In the latter, masses of fat—neutral fat and fatty acids—may bear a close resemblance to curds on macroscopic examination.

Vegetable cells (Fig. 23, *B, D, E, G, H, I, K, L*) are very varied in shape, and at times are mistaken for parasitic ova by inexperienced workers. The cells are less regular in shape and not so uniform in size as parasitic ova. Measurements are not necessary to demonstrate the great variation in size; it is quite obvious from inspection alone. Often the addition of a drop of Lugol's solution to the specimen will stain the vegetable cells, or at least some of them, blue. If the starch has been digested, this reaction is lost. The larger sheets of vegetable cells cannot be mistaken for anything else.

Vegetable spirals (Fig. 23, *J*) are the vessels of plants, which have

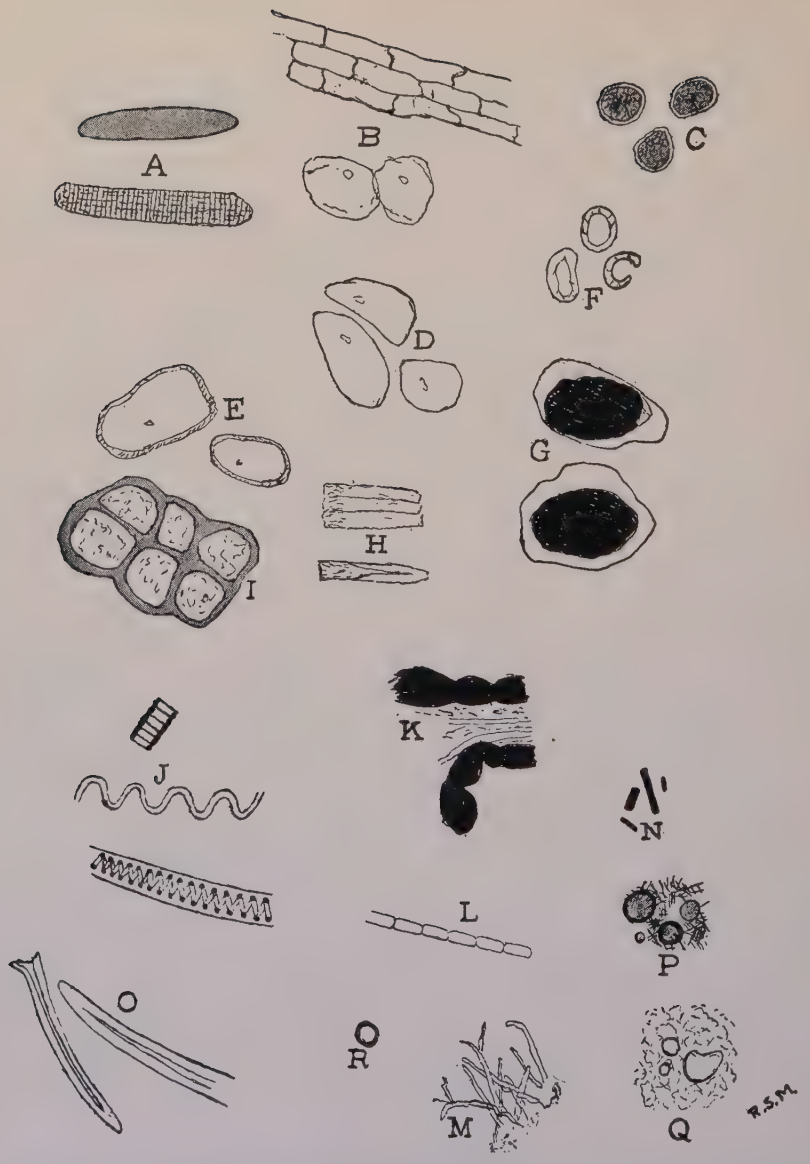

FIG. 23.—FECES (normal). *A*, muscle fibers (yellow), the upper one, partially digested, with rounded ends and loss of striations; *B*, onion cells, singly and *en plaque* (vegetable cells often stained yellowish from fecal pigments); *C*, lycopodium spores (for comparison of size of microscopic objects; average

Caption continued on p. 155.

escaped digestion in the intestine. When tightly coiled they present beaded borders with a latticed appearance between; when drawn out the spiral becomes evident.

Vegetable hairs (Fig. 23, *O*) are not infrequently mistaken for the embryos of parasites. They differ from all known embryos parasitic in man in having a perfectly homogeneous wall, devoid of cellular structure, with a central canal extending throughout. Furthermore, unlike living embryos, they possess no motility. They usually have a yellowish tint and are very refractive.

Starch (Fig. 22, *F*) granules are infrequent in the stools. Their usually oval shape, laminated appearance, and the iodin reaction identify them.

Unrecognizable *débris* is constantly seen in the stools.

Fat in the stools is discussed on p. 157.

Bacteria

Bacteria of all forms are extremely numerous in the stools, except in the case of breast-fed infants. With a few exceptions, little diagnostic importance is placed in their study. In typhoid fever the simplicity and accuracy of blood cultures make fecal examination for *Bacillus typhosus* superfluous, excepting in the case of typhoid bacillus-carriers with gall-bladder infections. In bacillary dysentery a search for the Shiga bacillus may be desirable; the same is true of Asiatic cholera. (For methods of detecting organisms such as these in the feces, the reader is referred to works on bacteriology.)

Tubercle bacilli are most readily found with the aid of the antiformin method (p. 212). It is necessary to cleanse the anus and surrounding parts in order to remove the smegma bacilli, as they are

diameter about 30 micra); *D*, potato cells (note variation in size and shape, characteristic of vegetable cells, and one of the differential points between these cells and parasitic ova); *E*, unidentified vegetable cells; *F*, calcium soaps; *G*, cells of the date; *H*, "palisade" cells (beans); *I*, cellulose framework of vegetable cells; *J*, vegetable spirals; *K*, banana; *L*, "chain" cells from dandelion. (*K* and *L* differ from dwarf tapeworm segments in being structureless uterus visible in tapeworm segments, and smaller than the parasite). *M*, fibrous connective tissue; *N*, crystals of bismuth suboxide; *O*, vegetable hairs or spines (at times mistaken for embryos of parasitic worms; the vegetable hairs, unlike embryos, possess no motility and are structureless); *P*, droplets of neutral fat, with needles of fatty acids and soaps; *Q*, milk curd (casein) with fat droplets; *R*, vegetable cell, unidentified, at times mistaken for ovum of Tænia Saginata or Tænia Solium (unlike the ovum, the vegetable cell has no radial striations in the membrane, and there are no hooklets within the cell). (All identified vegetable cells, and fibrous connective tissue after van Ledden Hulsebosch).

normally present in this part of the body. Since tubercle bacilli are frequently present in the feces of patients with pulmonary tuberculosis, due to the habit many adults—and all very young children—have of swallowing the sputa, they do not necessarily indicate an intestinal lesion. It is only when the bacilli are demonstrated in mucopurulent or bloody masses that a diagnosis of intestinal tuberculosis is probable. (For the method of staining tubercle bacilli see p. 211.)

Yeasts, often budding, may grow in the feces, and may be quite numerous. They may be present as a contamination after the stool has been passed.

Sarcinæ and *Oppler-Boas bacilli* (Fig. 22, p. 139) may be conspicuous in the feces. Their occurrence in large numbers is probably always secondary to pathological conditions in the stomach favoring their growth. Gram's stain should be used to demonstrate the long bacilli, for other bacteria are so numerous that they are less evident in the fresh specimen than they are in the stomach contents.

Cells

Epithelial cells of the intestinal mucosa desquamate continually. The single small, round, or oval nucleus is usually visible in the cell; if not, 3 per cent acetic acid should be added. Normally, epithelial cells are few in number. They may be well preserved, but are frequently swollen or otherwise degenerated. They are often found embedded in mucus.

Blood is never found in normal intestinal contents. It is only when the hemorrhage occurs in the lower part of the intestinal tract that the morphology of the cells is sufficiently well preserved to permit recognition on microscopic examination. Shadows of the red cells may be seen. Clots often contain erythrocytes in a good state of preservation. For the chemical tests for blood see pp. 146–149.

Pus cells—polynuclear neutrophilic leukocytes—in very small number, i.e., an occasional cell, are not pathological. If the cells are not degenerated beyond recognition, the distinguishing feature is the polymorphous nucleus, together with the finely granular protoplasm. The addition of dilute acetic acid may be necessary to demonstrate the nucleus. The cells may be free or embedded in mucus.

Eosinophilic leukocytes are never found in normal feces. They are most frequently, though not always,[12] associated with intestinal parasites

[12] Langstein, L. "Zur Kenntniss eosinophiler Darmkrisen im Säuglingsalter." *München. med. Wchnschr.*, 1911, LVIII, 623.

or protozoan infections. The cells are often found in particles of mucus, not infrequently blood-stained; *Charcot-Leyden crystals* are usually found among the eosinophiles.

Crystals

Crystals [13] are commonly seen in the stools. Those of *ammonio-magnesium phosphate* (Fig. 14, p. 88), are of frequent occurrence. They are often very imperfect. Crystals of *calcium oxalate* (Fig. 12, p. 83), and of *calcium phosphate* (Fig. 14, p. 88), are occasionally found. In addition, calcium salts of unknown acids may be precipitated in the feces as irregularly round or oval, bile-stained masses, at times with concentric rings. *Calcium soaps* (Fig. 23, *C*), are constantly present. *Cholesterin* (Fig. 13, p. 85), is rarely observed in crystalline form.

Fat.—Needlelike crystals of *fatty* acids and insoluble *soaps* (Fig. 23, p. 154), are always encountered on microscopic examination of the feces. The needles are short and slender, and are generally massed, so that the outline of the separate crystals is more or less obscure. At times they are extremely abundant, so much so that they may form the bulk of the stool. Fatty acids are soluble in alcohol and ether; soaps are insoluble. Fatty acids are further differentiated from soaps by the fact that their crystals melt to form droplets on warming. Morphological differentiation between the two may be impossible. Soaps may be present in the form of scales or long needles arranged in clusters. *Neutral fat,* in the form of droplets, may be found in any normal stool. The nature of the droplets may be clear from their varying size, high refractivity, and slightly greenish tint with strong illumination. To identify them with certainty, add to the specimen a drop of Sudan III or Scharlach R (saturated solution in 70 per cent alcohol), by which the droplets of neutral fat are stained orange to deep orange-red, the intensity of the color depending largely on the size of the droplet.

Charcot-Leyden crystals are never found in normal stools. They are always associated with the presence of eosinophilic leukocytes, though they may persist after the cells have disappeared. They are diamond-shaped, refractive bodies, which may be stained with eosin (Fig. 53, p. 209).

Bismuth suboxid (Fig. 23, *N*), appears in the stools in crystalline form after the administration of bismuth salts by mouth. The crystals

[13] The majority of these crystals have been described in the section on the urine, to which the reader is referred.

are black, irregular rhombs. They are frequently so abundant that the stool is dark or even black in color.

Hematoidin (Fig. 13, p. 85) crystals have been observed occasionally.

Intestinal Parasites [14]

In the Southern States and our island possessions intestinal parasites of one sort or another are the rule rather than the exception. Indeed, among the poorer classes of the population practically all are infected in certain localities. The free communication between all parts of the country is constantly disseminating the parasites, so that they are becoming of greater general importance each year. A description is given, therefore, of the important parasites, whose presence may be determined by fecal examination.

The low-power objectives are used in the examination of the feces for protozoa, ova, and embryos, the dry objectives of higher magnification being employed for final identification.

Protozoa—Rhizopoda

The majority of the intestinal protozoa have an encysted stage. The cysts correspond to the spores of bacteria, and are the means by which infection is transferred from one individual to another. They are much more resistant than the vegetative forms of the parasite, having a relatively high thermal death-point. The infected individual may continue to evacuate cysts in the feces long after the vegetative forms have disappeared, thus becoming a "carrier" of the disease. It is, therefore, of great importance to examine the feces for cysts of protozoa, as well as for the vegetative forms.

Method of Detecting Protozoal Cysts in the Feces.—The detection of protozoal cysts in the feces is much simplified by the use of the

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[14] The laboratory worker is advised to consult the Bulletins of the U. S. Public Health and Marine Hospital Service, Washington, D. C. Those issuing from the Department of Zoology by Stiles and his co-workers are invaluable to physicians. As reference works, the reader is referred to A. Castellani and A. J. Chalmers' excellent *Manual of Tropical Medicine* (3d ed.), N. Y., 1919; to H. B. Fantham, J. W. W. Stephens and F. V. Theobald's *The Animal Parasites of Man,* N. Y., 1920; to M. Neveu-Lemaire's *Précis de Parasitologie humaine* (5th ed.), Paris, 1921; and to R. Blanchard's *Traité de Zoologie médicale,* Paris, 1889.

iodin-eosin stain introduced by Donaldson.[15] The method, as slightly modified by Kofoid,[16] is as follows:

Solution (1). Saturated solution of eosin in normal salt solution.
Solution (2). Five per cent potassium iodid in normal salt solution, saturated with iodin.
Solution (3). Normal salt solution.

Two parts of solution (1), one part of solution (2), and two parts of solution (3) are mixed (fresh daily). The smear is prepared for microscopic examination by rubbing a minute bit of feces in a small drop of normal salt solution, and then in an adjacent drop of iodin-eosin stain. A single cover is placed on both drops, and the specimen is ready for examination.

Living flagellates and unstained cysts appear in the unstained part. In the stained area, the bacteria, fecal particles and yeasts (except the larger forms) stain at once with the eosin. Against the pink background, the protozoan cysts stand out sharply as bright spherules, which soon become tinged with iodin to varying tones of yellow, while their glycogen vacuoles, when present, become light or dark brown, depending on their size. The nuclei become more clearly defined as the iodin penetrates, especially in *E. coli* and *E. dysenteriae.* Plant hairs and a few mould spores are the only other objects taking the yellow color. Other débris takes on a more or less brownish tint. The "iodin cysts," shown by Kofoid to be the mononucleate cyst of *E. nana,* have a well-defined, round body, usually at one pole, which stains a very intense brown color (the glycogen vacuole). A vacuole in cysts of *E. coli* or *E. dysenteriae* may resemble this body, but it has no sharply defined outline, and is such a faint brown that its margins shade off into the surrounding color of the cyst. The *phycomycete spore,* also included by Wenyon in the "iodin cysts" (Kofoid), resists the eosin for a long time, but in some cases is soon tinged with iodin. Cysts of *Giardia intestinalis* and of *Chilomastix mesnili* stain yellow. *Blastocystis hominis* takes on a terra cotta color. Cysts of *Councilmania lafleuri* stain yellow.

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[15] Donaldson, Robert. "An easy and rapid method of detecting protozoal cysts by means of wet-stained preparations." *Lancet,* 1917, I, 571.

[16] Kofoid, C. A., Kornhauser, S. I., and Swezy, O. "Criterions for distinguishing the Endamœba of amebiasis from other organisms." *Arch. Int. Med.,* 1919, XXIV, 35.

TABLE SHOWING THE DISTRIBUTION OF INTESTINAL PARASITES IN MAN *

CASES OF INFECTION

	Total Cases Examined	Negative	Positive	Cestodes			Nematodes			Rhizopoda						Flagellata					Miscellaneous		
				Dibothriocephalus latus	Hymenolepis nana	Tænia saginata	Hookworm	Trichuris trichiura	Ascaris lumbricoides	Entamœba coli	Entamœba nana	Entamœba dysenteriæ	Entamœba gingivalis	Dientamœba fragilis	Amœba limax	Trichomonas intestinalis	Tricercomonas intestinalis	Embadomonas intestinalis	Chilomastix mesnili	Giardia intestinalis	Sporozoa	Blastocystis hominis	Phycomycete spore ("Iodin Cyst")
Overseas	2300	763	1537	0	10	0	160	136	26	473	675	297	1	1	3	3	3	4	97	131	7	784	194
Home Service	576	243	333	1	3	2	22	14	1	92	161	25	1	1	1	3	1	4	20	37	4	181	57
Combined	2876	1006	1870	1	13	2	182	150	27	565	836	322	2	2	4	6	4	8	117	168	11	965	251

PERCENTAGES OF INFECTION

Overseas	2300	33.1	66.9	0.0	0.4	0.0	6.9	5.9	1.1	20.5	29.3	12.8	0.1	0.1	0.1	0.1	0.2	0.2	4.2	5.7	0.3	34.1	8.4
Home Service	576	42.2	57.8	0.2	0.5	0.3	3.8	2.4	0.2	15.9	27.8	4.3	0.2	0.2	0.2	0.5	0.2	0.7	3.5	6.4	0.7	31.4	9.8
Combined	2876	34.9	64.7	0.03	0.45	0.06	6.3	5.2	0.94	19.6	29.0	11.2	0.06	0.06	0.1	0.2	0.13	0.28	4.0	5.0	0.4	33.2	8.3

* Kofoid, C. A., Kornhauser, S. I., and Plate, J. T. "Intestinal parasites in overseas and home-service troops of the U. S. Army," *Jour. A. M. A.*, 1919, LXXII, 1721; *also* Kofoid, C. A. "On the prevalence of carriers of Endamœba dysenteriae among soldiers returning from overseas service." *New Orleans M. and S. J.*, 1920, LXXIII, 4. (Table above from second reference.)

In making the preparation, avoid pressure on the cover glass, since cysts are easily ruptured and then stain red with the eosin.

The high-power dry objective is used in making the examination, and with artificial light, a blue glass is required to cut out the yellow light.

Entameba Dysenteriae.—*Entameba dysenteriae*[17] (Syn.: *E. histolytica*) (Fig. 24, *B, C*) found in the stools of those suffering with amebic dysentery, is a protozoan parasite belonging to the class Rhizopoda. When present in the stools, it is most readily found by selecting for examination particles of mucus, especially those which are bloodstained. The mucus may be obtained from the stools after administering a saline cathartic, if necessary, or by passing the rectal tube and removing the mucus which clings to the eye of the tube. In the absence of mucus, the fluid portion of the stool is examined, after giving the patient a saline cathartic. Since the amebae usually become quiescent soon after the specimen cools, it is absolutely essential that the material be examined at once, while still warm. A warm stage for the microscope is a great advantage, though not a necessity. In winter, placing the microscope on a radiator often furnishes enough heat to keep the parasites actively motile.

Entameba dysenteriae measures 0.010 to 0.035 to 0.070 mm. in diameter, though young forms which are smaller may be found. The majority of those seen in the stools are between 15 and 45 micra. The ameboid parasite possesses ectosarc and endosarc, which are well differentiated. The ectosarc has a peculiar greenish color, and, when thrown out to form a pseudopod, it presents an appearance suggesting that of ground glass. It is highly refractive. The endosarc or endoplasm is granular, and contains one to ten or more vacuoles—the younger forms usually only one—which are not contractile. If the intestinal lesions have been bleeding, many engulfed red corpuscles, often in a fair state of preservation, are seen in the endoplasm. The nucleus is not seen, as a rule. The parasite is possessed of ameboid motion which, in a fresh, warm specimen, is very active, rapid, and progressive, the parasite changing its position in a few seconds, so that it may cross the field of the

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[17] For an excellent discussion of the amebae of man see Craig, C. F. "The Parasitic Amœbæ of Man." Philadelphia and London, 1911. Also Kofoid, C. A., Kornhauser, S. I., and Swezy, O. "Criterions for distinguishing the Endamœba of amebiasis from other organisms." *Arch. Int. Med.*, 1919, XXIV, 35.

microscope. The ectoplasm is first protruded to form a pseudopod, and then the endoplasm flows into it. A whirling or circular motion of the endoplasm is not infrequently observed. When more sluggish, there may be simply protrusion and retraction of pseudopodia without change of position of the parasite.

The motility of the parasite is necessary for its recognition. In fact, it should be a rule in the diagnosis of amebic infections, to which no exceptions should be made, to refrain from calling any cell a vegetative form of ameba unless actual ameboid motion has been observed in an otherwise characteristic organism.

Simon [18] recommends staining the fresh specimen with dilute neutral red. A drop of dilute aqueous solution of the stain is allowed to run under the cover glass, or a minute particle of the powdered stain may be added to the specimen. There is a selective staining of the endoplasm of the parasite. Ameboid movements seem not to be interfered with. The organisms stand out very prominently.

Stained Preparations.—It is difficult to obtain satisfactory stained specimens of amebae. They may be stained with hematoxylin or with one of the Romanowsky stains. Brem [19] has had excellent results with a technic of his own. Cover slip preparations are made from the bloody mucus, and the specimens are then stained with Wright's, Hasting's, Leishman's, or Wilson's stain in the following manner:

1. The unfixed specimen is covered with four drops of the stain, which is allowed to act for 10 to 15 seconds. Since the stain is dissolved in absolute methyl alcohol, this fixes the specimen.

2. Add to the stain four drops of distilled water. At the end of one minute—

3. Add four more drops of stain. Again, at the expiration of one minute—

4. Add four drops of water. The specimen is thus covered with a mixture of stain and water in equal quantities. This is permitted to act for 10 to 30 minutes.

5. The specimen is now washed in distilled water. (The cover glass should be kept level, while a stream of water is directed against its surface. In this way the precipitated stain is washed or floated off;

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[18] Simon, C. E. "Clinical Diagnosis." 7th ed., p. 214, 1911. Philadelphia and New York.

[19] The method, devised by Dr. Walter Brem of Los Angeles, is unpublished and is given here with his kind permission.

dumping the staining mixture from the specimen causes the precipitate to adhere to it.) The specimen is quickly dried by holding it over a small flame or by blotting carefully, and is mounted in balsam.

The ectosarc of the amebae is stained dark blue, the endosarc a light blue. The nucleus takes a brilliant purplish-red color, and bacteria contained in the endosarc have a somewhat similar color. Phagocyted erythrocytes show a pinkish tint.

Resistant *encysted forms* are also encountered in the feces in *"carriers"* of the infection. The cysts of *Entameba dysenteriae* possess one to four nuclei (*Entameba tetragena*) and have a rather thin wall. They measure 6 to 15 micra in diameter (extreme measurements 5 to 20 micra). The cysts are readily demonstrable in the feces by means of Donaldson's iodin-eosin stain (p. 159). Encysted forms are found in the feces of 11.2 per cent of U. S. troops (Kofoid). (For the differentiation of cysts in the feces see tables on p. 164 and p. 170.) The thermal death-point of the cysts is 68° C.

Councilmania Lafleuri.— *Councilmania lafleuri* is a new intestinal ameba of man, recently described by Kofoid and Swezy.[20] They have found this protozoön in six cases. It was found in stools wholly free from *Entameba dysenteriae,* especially in blood and mucous strands. In the *vegetative stage* the ameba "is extraordinarily mobile, throws out perfectly hyaline, broadly rounded, single pseudopodia with expulsive suddenness, and travels rapidly through obstacles. Its cytoplasm is gorged with food vacuoles including bacteria, and cysts of *Chilomastix.* In the mucous strands it is frequently filled with red blood corpuscles."

"The *cysts* are exceptionally thick-walled, are double contoured, tend to be ellipsoidal or spheroidal rather than spherical, and range from 11 to 34 micra, generally 16 to 20 micra, in longest diameter. The cysts run through 1-, 2-, 4-, and 8-cell stages. One 12-cell cyst was encountered. Most of the cysts, except in liquid stools, are in the 8-cell stage when discharged in the feces. The structure of the nucleus differs from that in *E. coli.* The cysts are more difficult to stain. In the cysts the nuclear membrane is lightly encrusted with chromatin. . . . It is less often eccentric than in *E. coli.* The "glycogen" vacuole is present in the one to four-cell stages. It is spheroidal, central, and lobed as it disappears. It does not stain brown in iodin as in *E. coli,* though the

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[20] Kofoid, C. A. and Swezy, O. *"Councilmania lafleuri,* a new ameba of the human intestine." *Proc. Soc. Exp. Biol. and Med.,* 1921, XVIII, 310.

DIAGNOSTIC FEATURES OF CYSTS OF ENTAMŒBA DYSENTERIÆ, ENTAMŒBA NANA AND ENTAMŒBA COLI *

Criterion	*E. dysenteriæ*	*E. nana*	*E. coli*
Size	(5) 6–15 (20) micra.	(3) 5–12 (16) micra.	(11) 14–22 (35) micra.
Shape	Spheroidal, sometimes asymmetrically rounded.	Ellipsoidal in smaller forms, ovoidal in larger; frequently irregular, sometimes spherical.	Spheroidal, sometimes ellipsoidal, ovoidal or irregular.
Optical properties, unstained	Most highly refractive, glassy, often irregularly vacuolated; light grayish blue; nuclei rarely visible; glycogen mass not visible.	Less highly refractive, but with highly refractive granules, grayish blue; nuclei invisible; glycogen mass distinct.	Least highly refractive, homogeneous, porcellanous, grayish blue; nuclei faintly visible; glycogen mass faintly visible.
Stained with iodin-eosin (Donaldson's method)	*Cytoplasm* bright greenish yellow; coarsely vacuolated; in small races evenly but finely granular.	*Cytoplasm* greenish yellow, with numerous small, refractive vacuoles.	*Cytoplasm* coarsely, uniformly granular; yellowish brown.
	Nuclei distinct with highly refractive, thick border and distinct central granule.	*Nuclei* indistinct; with thin border and peripheral chromatin blob.	*Nuclei* very distinct, with thin granular borders and central granule.
	Glycogen diffuse; if massed, with vague limits.	*Glycogen* dense, in one or more large, sharply-defined masses, seldom central.	*Glycogen* central, with vague borders.
	Cysts stain red more quickly.	Cysts resist stain longer.	Cysts usually resist stain longer.
Nuclei: *number*	1. 30–45 per cent. 2. 13–30 per cent. 3. Rare. 4. 25–55 per cent.	1. Very common. 2. Less common. 4. Common in small race, very rare in large one.	1. Very rare. 2. Rare. 4. Very rare. 8. Very common. 16. Very rare.

DIAGNOSTIC FEATURES OF CYSTS OF ENTAMŒBA DYSENTERIAE ENTAMŒBA NANA AND ENTAMŒBA COLI*—*Continued*

Criterion	*E. dysenteriæ*	*E. nana*	*E. coli*
Structure	Central granule distinct; peripheral chromatin distributed rather evenly in small plaques.	No central granule; nuclear membrane indistinct; peripheral chromatin gathered in a single blob on the membrane.	Central granule distinct; peripheral chromatin in a few large plaques on the nuclear membrane.
Chromatoid substance	In one stout bar or several slender bars with rounded ends. Present in about 50 per cent of the cysts.	In several rounded or irregular masses. Rarely present.	In stout or slender splinters with square or sharp ends.
Cyst wall	Thin, distinct.	Variable, less distinct.	Thicker, very distinct.
Size races	Very evident. Small race at 7 micra, large race at 13–14 micra and one at 10 micra are the most frequent.	Very evident. One at 5–7 micra, one at 8 micra, 10 micra, and probably a still larger one at 12–15 micra, most frequent.	Less evident. Probably one at 15, one 18 micra, and one larger.

*Kofoid, C. A., Kornhauser, S. I., and Swezy, O. "Criterions for distinguishing the Endamoeba of amrebiasis from other organisms." *Arch. Int. Med.*, 1919, XXIV, 35.

cyst as a whole stains yellow. Some of the cysts are found to have a chromophile protoplasmic ridge which eventually pierces the cyst wall and forms the avenue of escape of the small amebulae. As a result of this discharge of amebulae, one finds cysts with varying numbers of nuclei (3 to 12)."

"*Councilmania* appears to be pathogenic, but more evidence is needed on this point. It occurs also in carriers."

Entameba Coli.—*Entameba coli* (Fig. 24, *A*), the non-pathogenic ameba of man, may be found in the feces of 2 to 65 per cent of healthy individuals after the administration of a saline purgative (Craig). It differs from the pathogenic amebæ in (*a*) its smaller size, the majority of the parasites measuring 10 to 30 micra in diameter; (*b*) lack of

sharp definition between ectosarc and endosarc; (*c*) presence of an easily recognizable nucleus, as in tetragena; (*d*) its opaque grayish color, especially well seen in the younger forms; (*e*) the small number of vacuoles and absence of erythrocytes in the endosarc (rarely a few red corpuscles may be engulfed when they are present in the feces); (*f*) the very sluggish ameboid movements with little, if any, change in position in the specimen; and, finally, (*g*) its reaction to the Romanowsky stains, the ectosarc taking a light blue, the endosarc a dark blue, color—just the reverse of the conditions seen in *dysenteriae.*

Encysted forms, by means of which the infection is transmitted, are

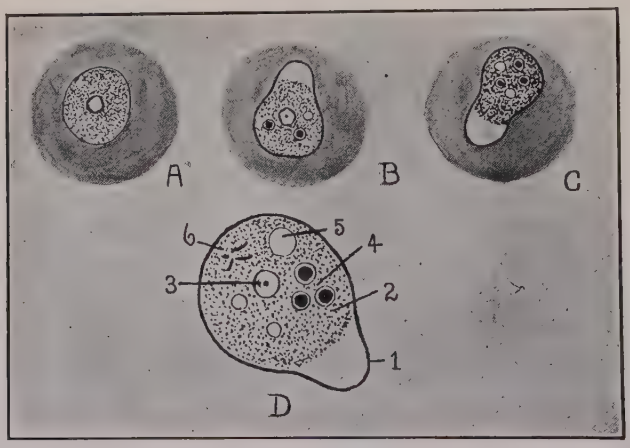

Fig. 24.—Parasitic Amebae.—*A*, Entameba coli; *B*, *C*, Entameba dysenteriae; *D*, diagrammatic; 1, ectoplasm; 2, endoplasm; 3, nucleus, nuclear membrane, centriole; 4, erythrocytes; 5, vacuole (adapted from Craig).

encountered in the feces. The cysts are larger than those of *Entameba dysenteriae,* have a thicker wall, and *eight* nuclei. The cysts of *Entameba coli* are 14 to 22 micra in diameter (extremes 11 to 35 micra). They are demonstrable by Donaldson's method (p. 159). This protozoon has been found in the feces in 19.6 per cent of U. S. troops. (Kofoid). (See tables, pp. 164, 170.) The thermal death-point of the cysts is 76° C.

Entameba nana (Endolimax nana).—*Entameba nana* has been found in the stools in 29 per cent of troops in the U. S. army (Kofoid). (*Entameba coli* 19.6 per cent; *Entameba dysenteriae* 11.2 per cent, see table, p. 160.) It is non-pathogenic, but its importance lies in the possibility of confusing it with *Entameba dysenteriae. Entameba nana*

is usually found in the stools as cysts, but after a saline the vegetative forms may be present; the pseudopodia are broad and hyaline. The amebae are small, 5 to 10 micra in diameter. The nucleus has a large, irregularly shaped chromatin mass. The amebae are usually vacuolated and contain bacteria.

The *cysts* are usually oval (resembling Giardia, but without the interior lines), and have thin walls. As in the vegetative forms, the cyst contains but one nucleus (rarely two and exceptionally four). When the cysts are round, the nucleus distinguishes them from cysts of *Entameba dysenteriae,* which usually have four nuclei, and *Entameba coli,* which, in the encysted form, has eight nuclei. The cysts of *Entameba nana* measure 5 to 12 micra (extremes 3 to 16 micra). They are demonstrable in the feces by Donaldson's method (p. 159). The differentiation of these cysts from other ameba cysts is indicated in Kofoid's table (p. 164). (See also table, p. 170.) The thermal death-point of the cysts is 64° C.

Resistant encysted forms, by which the infection is transmitted, have been described for the three species of amebae considered above.

"Iodin Cysts."—The so-called "iodin bodies" or "iodin cysts" (*Iodameba wenyoni*) have caused some confusion in fecal examinations. Kofoid and his co-workers[21] have made a careful study of the cysts and have come to the conclusion that at least two different cysts have been included under "iodin cysts." The large, mononucleate cysts of *E. nana,* rich in glycogen, correspond to the cysts described by Wenyon containing a mass of iodophilic material. In addition to this, Kofoid has recognized among the "iodin cysts" a *phycomycete spore* of unknown relationships. The latter are cysts which are homogeneous and quite regularly spherical, measuring 10 to 24 micra in diameter (extremes 7 to 30 micra). The cytoplasm is homogeneous, there are no nuclei, and rarely one or more eccentric or central, empty, nuclearlike vacuoles. There may be processlike protrusions of the cytoplasm. In iodin-eosin, they resist the stain for a long time, but in some cases are soon tinged with iodin. They were found in 8.3 per cent of U. S. troops (see table, p. 170).

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[21] Kofoid, C. A., Kornhauser, S. I., and Swezy, Olive. "Structure and systematic relationships of the 'iodin cysts' from human feces." *The Military Surgeon,* 1919, XLV, 30.

Flagellata

Trichomonas Intestinalis.—*Trichomonas intestinalis*[22] (Fig. 25) is a pear-shaped body measuring 0.010 to 0.015 mm. long and 0.003 to 0.004 mm. wide (Braun). The posterior end of the parasite is pointed. Four flagella are attached to the anterior, rounded end, and there is an undulating membrane running from the point of insertion of the flagella to the posterior extremity of the organism. The body of the parasite is quite refractive and has a greenish, glasslike appearance in a strong light. Its motility is often so extreme that many of the details of structure of the parasite are seen with difficulty, if at all. The fluid portion of a perfectly fresh stool should be taken for examination. *Trichomonas intestinalis* is probably non-pathogenic. Encysted forms have not been found. Trichomonas was encountered in 0.2 per cent of U. S. troops.

FIG. 25.— TRICHOMONAS INTESTINALIS. ×about 1200 (after Freund).

Chilomastix Mesnili (*Tetramitus Mesnili*).—This parasite, like the preceding, is a flagellate. It possesses no undulating membrane. The parasite is generally pear-shaped, measuring 11 to 18 micra in length, if the caudal appendage is included, and some 1.6 to 4 micra less, if this is excluded (Castellani and Chalmers). There are three long and slender flagella projecting anteriorly. There is a prominent, long, slitlike cytostome situated anteriorly, within which there is a flagellum. The non-flagellate end is much attenuated. The parasite has been reported as a cause of diarrhea. It is found in the large intestine, especially in the caecum. Small encysted forms occur, demonstrable by the iodin-eosin stain. The *cysts* measure 5.6 × 7 micra, are oval, and are characterized by the presence of a chromatic rod. The thermal death-point of the cysts is 72° C.

Giardia Intestinalis.—*Giardia intestinalis* (Syn.: *Lamblia intestinalis*), (Fig. 26), a third non-pathogenic flagellate parasitic in man, appears to be of less frequent occurrence than the two preceding. Viewed ventrally or dorsally, the parasite is pear-shaped. It is provided with a cup-like depression or excavation near its anterior extremity, by means

[22] Freund, H. "Trichomonas hominis intestinalis; a study of its biology and its pathogenicity." *Arch. Int. Med.*, 1908, I, 28.

of which it attaches itself to the epithelial cells of the mucosa of the small gut. The length of the parasite varies between 0.010 and 0.021 mm., the breadth between 0.005 and 0.012 mm. It is provided with eight flagella 0.009 to 0.014 mm. long, arranged in pairs. The first pair arises from the anterior, the second and third pairs from the posterior end of the cuplike depression, while the fourth pair is attached to the posterior pointed extremity of the body. The protoplasm is finely granular. The nucleus may be seen at times beneath the depression. If intestinal peristalsis is normal or diminished, the forms just described may not be observed in the stools. In their stead *encysted forms,* egg-shaped, very transparent, showing the lamblia within and measuring 6-7 × 13-14 micra, are evacuated with the feces. The thermal death-

FIG. 26.—GIARDIA INTESTINALIS. Ventral and lateral views; on the intestinal epithelial cells; dead; and encysted (after Grassi and Schewiakoff, from Braun).

point of the cysts is 64° C. (See table, p. 170.) Acceleration of peristalis from whatever cause may lead to the presence of the active stage of the parasite in the stools. It was found in 5 per cent of troops in the U. S. army (Kofoid), and in about 20 per cent of healthy children.[23]

Blastocystis hominis is an organism, supposed to belong to the moulds, which may be mistaken for encysted flagellates. It has been found in 33.2 per cent of troops in the U. S. army (Kofoid). It has a large central vacuole with a refractile, narrow rim, which contains one or more nuclei. With Giemsa's stain, the central part is very faintly stained, while the rim is deep blue. (See table, p. 170.)

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[23] Maxey, K. F. "*Giardia* (*Lamblia*) *intestinalis,* a common protozoan parasite of children." *Johns Hopkins Hosp. Bull.,* 1921, XXXII, 1721.

TABLE OF COMPARISON OF PROTOZOAN CYSTS IN THE FECES *

Parasite	Size	Nuclei	Thermal Death-point	Appearance
Entameba dysenteriae	10–14 micra (extremes 7–20)	Four, often one or two	68° C.	Spherical, greenish, very refractive; glycogen mass not visible. Pathogenic.
Entameba nana	5–12 micra (extremes 3–16)	One usual, 4 common in small race.	64° C.	Spheroidal, ovoidal, or irregular; less highly refractive; glycogen mass distinct.
Entameba coli	14–22 micra (extremes 11–35)	Eight.	76° C.	Spheroidal, at times ovoidal or irregular; least highly refractive; glycogen mass faintly visible.
Councilmania lafleuri	16–20 micra (extremes 11–34)	One to 12, usually 8.	....	Ellipsoidal or spheroidal, exceptionally thick-walled, double-contoured. Glycogen vacuole present in 1- to 4-cell stage; does not stain brown in iodin, as in *E. coli*. Probably pathogenic.
Giardia intestinalis	6–7×13–14 micra		64° C.	Egg-shaped, very transparent, showing the parasite within it.
Chilomastix mesnili	7×5.6 micra		72° C.	Oval, characterized by the presence of the chromatin rod.
Blastocystis hominis	3–20 micra		....	More or less spherical, capsule more delicate than those of *Entamebae;* contains a large vacuole.
"Iodin Cysts"	(See text, page 167).			
(*Phycomycete spore*)	10–24 micra (extremes 7–30)			Spherical, cytoplasm homogeneous, with alveolar vacuolations at times. Thin-walled protrusions of cytoplasm may be observed at times.

* Adapted, with modifications and additions, from Castellani, A., and Chalmers, A. J. *Manual of Tropical Medicine.* (3d ed.), N. Y., 1919, p. 1833.

Infusoria

Balantidium Coli.—*Balantidium coli* (*Paramecium coli*) [24] is a protozoön which may be pathogenic for man, producing a disease somewhat analogous to amebic dysentery with ulcers in the colon. It is very common in hogs. The parasite (Fig. 27) is oval, 0.060 to 0.100 mm. in its long diameter by 0.050 to 0.070 mm. The anterior end is less pointed than the posterior. A funnel-shaped peristome is situated anteriorly, about which are numerous cilia. Cilia are also conspicuous on the surface of the parasite. Ectosarc and endosarc are visible. The latter is glandular and may contain fat droplets, bacteria, mucus, at times erythrocytes and pus corpuscles. Two or more contractile vacuoles are found in the endosarc. A macronucleus, rather kidney-shaped, and a round micronucleus, situated posteriorly, may be seen. Encysted forms, by means of which the infection is transferred, are described.

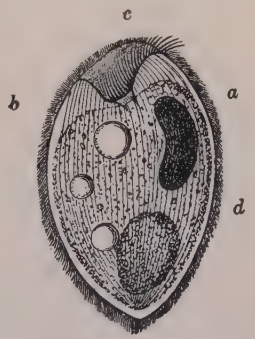

FIG. 27.—BALANTIDIUM COLI. *A*, nucleus; *B*, vacuole; *C*, perostome; *D*, food mass (after Leuckart, from Braun).

Balantidium coli, common in hogs, is an uncommon parasite of man. Cases of dysentery, due to this organism, are reported; and in the chronic form of the infection, the blood picture (including megaloblasts) and the spinal cord changes characteristic of pernicious anemia may develop.[25]

Dipterous Larvae.—Larvae of dipterous insects [26] (flies) may appear in the feces (myiasis). "They are easily recognized. The ringed, cylindrical body, from ½ to 1 inch in length, according to the species, broad at one end, tapering at the other, and usually beset with little spines or hairs, is sufficiently diagnostic" (Manson [27]). It is particularly important that the specimen for examination be fresh for obvious reasons.

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[24] Bowman, F. B. "The pathogenesis of *Balantidium coli.*" *Jour. A. M. A.*, 1911, LVII, 1814.

[25] Logan, A. H. "*Balantidium coli* and pernicious anæmia: Report of four cases." *Am. Jour. Med. Sci.*, 1921, CLXII, 668.

[26] Gilbert, N. C. "Infection of man by dipterous larvæ, with report of four cases." *Arch. Int. Med.*, 1908, II, 226.(Literature.)

[27] Manson, P. "Tropical Diseases." London, 1900, p. 603.

Parasitic Worms

Infection with parasitic worms is diagnosed by finding in the feces the adult parasite, its embryos, or ova. In light infections, diagnosis is facilitated by special methods of concentrating the ova, of which the following are the more important:

1. **The Brine-loop Method of Kofoid and Barber.**[28]—This is the best of the special methods. A large fecal sample (the entire stool, if the receptacle is large enough) is mixed thoroughly with concentrated brine with a wooden stick in a vessel of 2 to 3 ounces capacity (a paraffined paper can, destroyed after using, is used by the authors). The proportion of stool to brine should be about 1 : 3. The specimen must now be allowed to stand about 1 hour. The coarser floating particles are skimmed off with a spoon (which should be scrubbed and rinsed in hot water after using).

Prepare a wire loop about ½ inch in diameter. (Galvanized wire from screens is satisfactory and inexpensive.) After an hour, the *parasitic ova* and *cysts of amebae* and of *Giardia* and *Chilomastix* will have risen to the surface, and are found floating in the scum on the brine. Sink the loop in the brine and raise it slowly. The surface film or scum will adhere to the loop, and is now transferred to a glass slide. About ten (10) loopfuls will skim the surface, and may be placed on a glass slide. The preparation is examined wet without a cover glass, using low power and cutting off most of the light.

Caution.—Fewer ova are found when the surface film is examined at once (before all the ova have floated to the surface), and also when examination is delayed 24 hours.

Ova of the *hookworm, Ascaris lumbricoides, Oxyuris vermicularis,* the *whipworm, Taenia saginata, Taenia solium, Hymenolepis nana, Hymenolepis diminuta, Dipylidium caninum* and of *trematodes* are floated up by the brine without distortion in 1 to 2 hours. Cysts of *Entameba coli, Entameba dysenteriae, E. nana, Chilomastix,* and *Giardia* (*Lamblia*) *intestinalis* are also floated.

2. **Stiles' Method of Washing and Sedimenting.**[29]—"Take one to two ounces of feces, mix with water, and place in a large bottle, retort,

[28] Kofoid, C. A. and Barber, M. A. "Rapid method for detection of ova of intestinal parasites in human stools." *Jour. A. M. A.*, 1918, LXXI, 1557.

[29] Stiles, C. W. "Report on the prevalence and geographic distribution of hookworm disease in the United States." *Bull. No. 10,* Hyg. Lab., U. S. Pub. Health and Mar. Hosp. Serv., Wash., 1903, p. 85.

jar, or any other receptacle; add enough water to make from a pint to two quarts, according to the amount of feces; shake or stir thoroughly and allow to settle; pour off the floating matter and the water down to near the sediment; repeat the washing and settling several times, or as long as any matter will float. The last time this is done use a bottle or graduate with a smaller diameter, and, when the material is thoroughly settled, examine the fine sediment. It will be found that the eggs have settled more numerously in the fine sediment than in the coarse material."

3. **Centrifugalization.**—Simple centrifugalization of the diluted feces often gives disappointing results. The essentials of the method, as given by Dock and Bass,[30] follow: "The feces should be diluted and well mixed with ten or more times their bulk of water. This should be strained through two or three layers of gauze in a funnel to remove the coarse particles. The exact length of time necessary to centrifuge, in order to throw most of the eggs suspended in water to the bottom of the tube, should be determined by experimenting with a known specimen that has already been washed once or twice and contains many eggs. This must be determined with the particular centrifuge used. . . . As the first diluted feces are much thicker than the washed feces and eggs on which the working time of the centrifuge has been determined, the eggs will go down somewhat slower the first time. It is, therefore, a good plan to centrifuge double time at first. If, for example, the working time of the centrifuge is four seconds, the first centrifuging should be eight seconds. This throws to the bottom most things heavier than eggs —like crystals, sand, large vegetable cells, etc.—and all eggs present. There remain suspended in the supernatant fluid nearly all bacteria and fine particles, and many coarse particles lighter and more irregularly shaped than eggs. If the centrifuge is run longer, many of these go down, which is, of course, undesirable. Pour off this fluid and two-thirds, and often more, of the feces are removed by this washing. Refill the tube to about three-fourths its capacity, shake up thoroughly, and centrifuge again, running now only the working time of the centrifuge. It is important not to centrifuge longer than the working time of the centrifuge, as many fine and light particles would otherwise be thrown down. Considerable material remains suspended and may be removed by pouring off the supernatant fluid. Again the tube is filled, shaken,

[30] Dock, G. and Bass, C. C. *Hookworm Disease*, etc. St. Louis, 1910, pp. 172 *et seq.*

TABLE INDICATING MODE OF INFECTION AND DIAGNOSIS IN THE COMMONER INTESTINAL PARASITES

NEMATODES

Parasite	Infection	Diagnosis
Hookworm (*Necator americanus*) (*Ankylostoma duodenale*)	Through skin (of feet usually) by strongyloid larvæ, or by swallowing larvæ.	Ova in feces.
Strongyloides stercoralis	Through skin by filariform larvæ or by swallowing the larvæ.	Rhabditiform embryos in feces.
Ascaris lumbricoides	Swallowing ova containing embryos (green vegetables, water, etc.).	Ova in feces. Adult worm in feces, vomitus, etc.
Trichuris trichiura (Whip-worm)	Swallowing ova containing embryos.	Ova in feces.
Oxyuris vermicularis	Swallowing ova containing embryos.	Ova in scraping from perineum; from dirt under finger nails of the child; less often ova and parasites in stools.
Trichinella spiralis	Eating undercooked "measly" pork (encysted embryos).	Embryos in blood or spinal fluid; "harpooning" muscle.

CESTODES

Tenia saginata	Eating undercooked, infested beef (*Cysticercus bovis*).	Ova and proglottids in feces.
Tenia solium	Eating undercooked, infested pork (*Cysticercus cellulosae*).	Ova and proglottids in feces.
Dibothriocephalus latus	Eating undercooked, infested fish.	Ova and proglottids in feces.
Hymenolepis nana	Swallowing ova.	Ova in feces; proglottids (very small) at times.

TREMATODES

Schistosoma mansoni	Drinking, or bathing in, water containing embryos.	Ova in feces.
Schistosoma japonicum	Drinking, or bathing in, water containing embryos.	Ova in feces.
Schistosoma hematobium	Drinking, or bathing in, water containing embryos.	Ova in *urine*.
Paragonimus ringeri	Eating raw, infested crabs.	Ova in *sputum* or feces.

and centrifuged a proper length of time, and generally this will be sufficient for practical purposes. A part or all of the sediment is removed with a pipette, spread out on a slide, and examined for eggs. It consists of crystals, sand, and heavy, coarse food particles, and eggs, if present. . . . Great care must be exercised to clean the centrifuge tubes before using them after they have had eggs in them. A proper centrifuge brush is serviceable. The method . . . permits the finding of eggs when less than half a dozen laying females are present, and often when only one is present. It is of additional service because it permits at the same time diagnosis of infections with many other worms by which fewer eggs are laid, such as the tenias, oxyuris, bothriocephalus, etc."

Nematodes

Nematodes,[31] round worms, constitute a large class, a number of which are parasitic in man. Their anatomy and biology, though of great interest, are touched upon in the following pages only in so far as they are of diagnostic significance.

Necator Americanus.—*Necator americanus* (*Uncinaria americana*), the New World hookworm, was first described by Stiles[32] in 1902. Together with its cousin of the Old World, it is by far the most important nematode parasitic in man in this country. It is the causative agent of the disease uncinariasis or ankylostomiasis.

Diagnosis of infection with *Necator americanus* is made by examination of the stools for the ova of the parasite. The specimen of feces for examination should be fresh; in older specimens—24 to 48 hours—the eggs may have hatched, in which case the embryos, much like those of *Strongyloides stercoralis,* may be observed. The specimen is examined with low power objective. All doubtful objects are examined with higher magnification. The ovum of the hookworm is characteristic, and, when once seen, can scarcely be mistaken for other bodies in the stool.

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[31] Ova of the commoner nematodes, drawn to scale, are shown in Fig. 37, p 185.

[32] Stiles, C. W. (a) "A new species of hookworm (*Uncinaria americana*) parasitic in man." *Amer. Med.,* 1902, III, 523. (b) "The significance of the recent American cases of hookworm disease (uncinariasis or ankylostomiasis) in man." *Reprint, 18th Ann. Rep.,* Bureau Anim. Industry (1901). Wash., 1902. (c) "Report on the prevalence and geographic distribution of hookworm disease (uncinariasis or ankylostomiasis) in the United States." *Bull. No. 10,* Hyg. Lab., U. S. Pub. Health & Mar. Hosp. Serv., Wash., 1903, pp. 1-122.

The *ova* (Fig. 28) of *Necator americanus* are oval and possess a clear, colorless shell, which measures 0.064 to 0.076 mm. by 0.036 to 0.040 mm. (Stiles). The outline of the egg is sharp and clearly defined. Inside the shell is the yolk, which is unsegmented when deposited by the female in the intestines, but usually presents two, four, or eight segments or cells, sometimes more, by the time it is evacuated with the feces. The yolk is dark gray or brownish-gray and finely granular; usually a lighter area representing the nucleus may be observed near the center of the yolk cells, especially when their number is eight or less; as the cells multiply, the decrease in size makes this area less conspicuous.

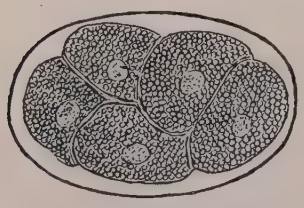

FIG. 28.—OVUM OF NECATOR AMERICANUS. ×460.

In examining a preparation for hookworm (or other) ova, a mechanical stage is a great convenience. About one-half the area of the usual 3 × 1-in. slide should be covered with the diluted feces, and before rendering a negative diagnosis, ten such specimens should be examined (Stiles). ". . . To stop after finding a few hookworm eggs is not good practice. The examination should be continued to find, if present, eggs of other parasites, which are likely to be present in small numbers, and to get some idea of the number. When less than ten female worms are present, there may be an average of less than one egg to a slide" (Dock and Bass [33]).

The *adult worms* of *Necator americanus* (Fig. 29) are found in the stools only after treatment. They are small, whitish, grayish, or reddish-brown in color, and have the anterior end curved dorsally to form a hook. The males measure 6 to 10 mm. in length, the females 8 to 15 mm. The stools, collected after treatment, are placed in a bucket or other suitable receptacle, stirred with several times their volume of water, and allowed to settle a few minutes, when the supernatant fluid is poured off. The washing is repeated several times, and, finally, the sediment is transferred to a plate, preferably with a black background, and the worms looked for. They may be identified by examination under the microscope.

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[33] Dock, G., and Bass, C. C. *Hookworm Disease. Etiology, pathology, diagnosis, prognosis, prophylaxis, and treatment.* St. Louis, 1910. (An excellent discussion of hookworm disease in all its medical aspects, to which the reader is referred.)

Ankylostoma Duodenale.—*Ankylostoma duodenale,* the Old World *hookworm,* is of frequent occurrence in this country, often associated with *Necator americanus.* The methods of diagnosis are those described for *Necator americanus.* The *ova* (Fig. 28) are identical in appearance. Measurements show, however, that they are a little smaller than those of the New World variety, measuring 0.052 to 0.061 by 0.032 to 0.038 mm. The differences are so slight that simple microscopic inspection

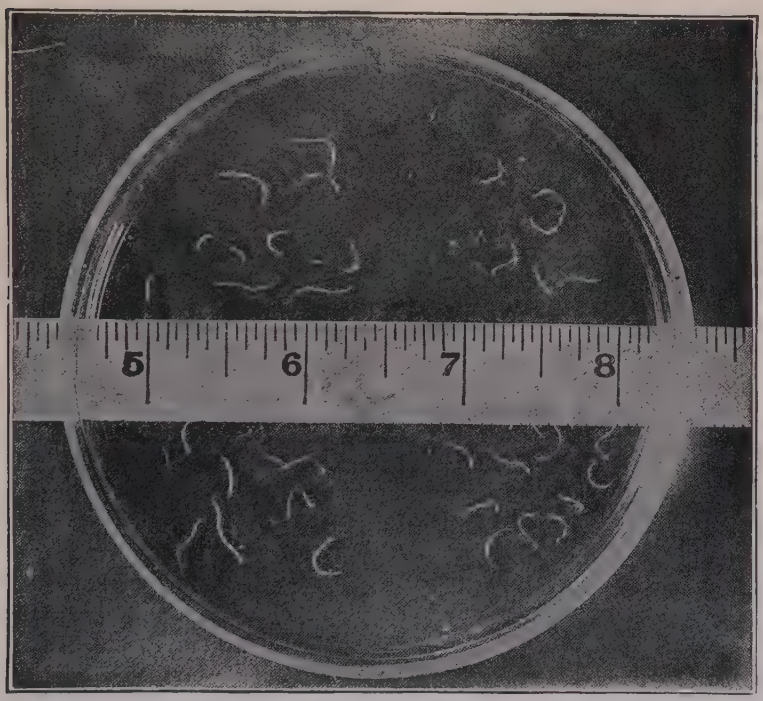


FIG. 29.—NECATOR AMERICANUS. Upper half males, lower half females. Inch measure (after Dock and Bass).

does not suffice for the separation of the two. The *adult parasites* are a trifle larger than *Necator americanus.* The males are 8 to 11 mm. long and 0.45 mm. wide; the females 10 to 18 mm. long and 0.6 mm. wide. There are certain well marked peculiarities by which the two species of hookworm may be differentiated.

The hookworms, *Necator americanus* and *Ankylostoma duodenale,* are very prevalent in the Southern States, and are, in fact, found in all

tropical and sub-tropical countries, and in many temperate climates. In many mines, contamination of the soil leads to widespread hookworm disease among the workers.

Soil becomes infected through contamination with feces containing the ova; embryos develop from the ova, and eventually arrive at a stage which is infective for man. Infection occurs either from swallowing the embryos (contaminated water, green vegetables) or, much more commonly, by way of the skin, particularly the skin of the feet. The infection is usually contracted by an individual through walking with bare feet through infected soil. The embryos attach themselves to the skin, which they pierce, causing the condition known as "ground itch." Entering the lymphatics, the embryos pass *via* the thoracic duct to the right heart, thence to the capillaries of the lungs. Piercing the wall of the capillaries, they enter the lung alveoli, make their way up the bronchial tree, through trachea and larynx, to the pharynx. Through the act of swallowing, the embryos gain entrance to the intestines of man, where they develop into the adult worms. The cutaneous infection was first demonstrated by Looss.

Infection with hookworm is the commonest cause of anemia in the South. The anemia is of varying degree; it may be extreme, and is usually of the chlorotic type, with low color index. Eosinophilia is inconstant. In a study of nineteen severe cases, Ashford, in Porto Rico, obtained the following averages: Red cells 1,776,000; hemoglobin 21 per cent; color index 0.6; leukocytes 7,000; eosinophiles 10.3 per cent. Boycott has shown that, before a severe anemia occurs, there may be a marked increase in the number of leukocytes (20,600 to 56,000), with marked eosinophilia (56 to 66 per cent), with hemoglobin above 80 per cent. With the development of severe anemia, the leukocyte counts falls, and there may be a leukopenia (Castellani and Chalmers).

Strongyloides Stercoralis.—*Strongyloides stercoralis* (*Strongyloides intestinalis*), the parasite of Cochin China diarrhea, first reported in this country by Strong [34] and Thayer,[35] has proved to be widely spread through the Southern States, as Thayer predicted. Diagnosis of infection is made by the finding of the actively motile *rhabditiform embryos* in the stool.

[34] Strong, R. P. "Cases of infection with Strongyloides intestinalis (first reported occurrence in North America)." *Johns Hopkins Hosp. Rep.*, 1902, X, 91.

[35] Thayer, W. S. "On the occurrence of Strongyloides intestinalis in the United States." *Jour. Exp. Med.*, 1901, VI, 75.

Perfectly fresh feces should always be used for examination. If the specimen is kept too long, the embryos may die or may change into the filariform larvæ. If the stool is formed, fluid about it may contain the embryos, or they may be looked for in a preparation of the diluted feces.

FIG. 30.—THE RHABDITIFORM EMBRYO OF STRONGYLOIDES STERCORALIS. ×460.

Since this often fails, even with heavy infections, it is better practice to give a saline cathartic, if necessary, and examine the fluid stools. The embryos (Fig. 30) are 0.450 to 0.600 mm. long and 0.016 to 0.020 mm. thick (Blanchard), and have a characteristic wriggling or squirming motion in the fresh stool. They are grayish-white and quite refractive. They possess a rhabditiform or bottle-shaped esophagus. Embryos which have died are, of course, less conspicuous, but, if well preserved, are characteristic. If the infected stool be kept for one to two days under suitable conditions of light, moisture, temperature, and oxygen supply, the rhabditiform embryos may develop into the filariform larvæ, the infecting stage of the parasite.

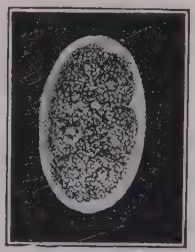


FIG. 31.—OVUM OF STRONGYLOIDES STERCORALIS. Drawn with Leitz obj. No. 7, ocular No. 3 (after Thayer).

Ova of Strongyloides stercoralis (Fig. 31) are extremely rare in the feces. They resemble the ova of the hookworm. In one of his cases Thayer found two eggs on daily examination of the stools over a period of several months.

Hookworm embryos which have developed in the stools (twenty-four to forty-eight hours after passage) may be mistaken for the embryos of

Strongyloides stercoralis; the former never occur in the freshly evacuated feces. They are most readily differentiated by the fact that the buccal capsule (Fig. 32) is very short in the embryo of *Strongyloides stercoralis,* relatively long in the hookworm embryo (Stiles). In case of doubt, a perfectly fresh stool should be obtained after a saline cathartic, when ova without embryos are found with hookworm infection alone; with double infection, both embryos and ova are seen.

Students often confuse *plant or vegetable hairs* with (dead or inactive) embryos. The former are distinguished by a straight central canal with hyalin, refractive walls of quite uniform thickness and devoid of finer structure.

The adult parasite, which inhabits the small intestine, is probably

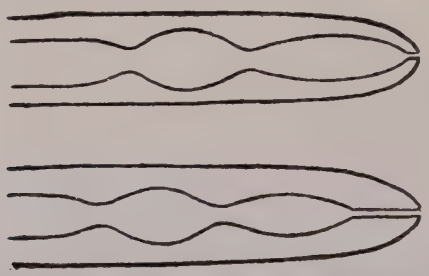


FIG. 32.—THE RHABDITIFORM EMBRYO OF *Strongyloides stercoralis* (1) AND THE EMBRYO OF THE HOOKWORM (2). Showing the difference in the length of the buccal capsule. *Diagrammatic.*

a parthenogenetic female, and is a rarity in the stool. It is quite minute —2.2 mm. long and about 0.034 mm. thick.

Infection with *Strongyloides stercoralis* occurs by swallowing the filariform larvae directly, or, more commonly, by the cutaneous route, as in the case of the hookworm (q. v.), the embryos following the same channels to the intestine as the hookworm embryos.

Anemia is present with heavy infections. The blood picture is quite similar to that seen in hookworm disease; the color index is low, the leukocytes normal or decreased in number, and eosinophiles usually increased, even to 45 per cent (personal observation). Except for the eosinophilia, the appearance of the stained blood *may* resemble closely that seen in chlorosis.

Oxyuris Vermicularis.—*Oxyuris vermicularis* (*Ascaris vermicularis*), the common pinworm or seatworm, is a small nematode, the males measuring 3 to 5 mm. long with the posterior end curved, the females

about 10 mm. long and 0.6 mm. thick. The *ova* (Fig. 33) are flattened on one side, measure 0.016 to 0.025 by 0.050 to 0.055 mm. (Castellani and Chalmers, and Neveu-Lemaire), and have a clear, thin shell. The ova are deposited with the embryos already developed within the shell. It is necessary to recall the fact that the gravid females habitually wander from the region of the cecum and appendix to the rectum, anus, perineum, etc., in order to appreciate clearly the reasons for the methods of diagnosing infection. The crawling of the worms over the skin causes itching, so that the patient usually scratches the affected part.

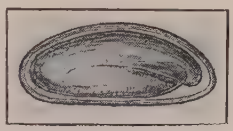


FIG. 33. — OVUM OF OXYURIS VERMICULARIS. ×460.

The presence of pinworms may be determined [36] by (1) examining the feces for the adult worms (p. 176). Usually only females are found. The material for examination is best obtained by an enema given in the evening. (2) The worms may be seen in the crotch, especially if the child be examined during the restless period after retiring. (3) Microscopic examination of the scrapings of the skin about the anus or of dirt from the finger nails (the ova being picked up in scratching the perineum) may reveal the ova. (4) Eggs may be found in the feces. Fecal examination for the eggs of the parasite is the least trustworthy of the methods of diagnosing infection. Stiles' statement, which is agreed to by experienced observers, should be borne in mind. He says: "The writer's experience is that the eggs may be found in fecal examination in some cases in which pinworm is not even suspected; but that a negative examination is not of much value." Examination for the mature worms or for the ova in the scrapings of the perineum or finger nails gives more reliable results.

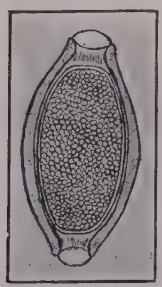


FIG. 34.—OVUM OF TRICHURIS TRICHIURA. ×460.

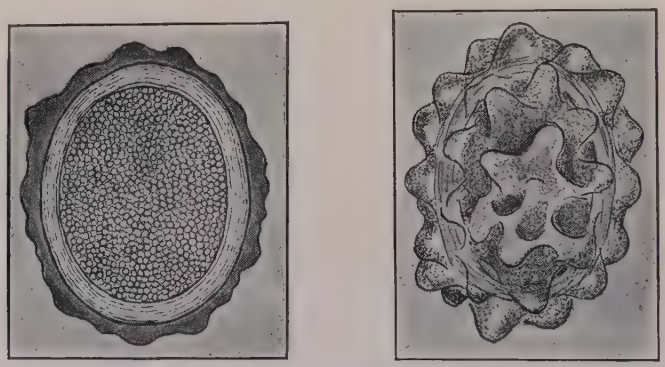
Trichuris Trichiura.—*Trichuris trichiura* (*Trichocephalus dispar*), the whipworm, is rarely seen in the feces. It may be found after treatment has been administered. The males are 35 to 45 mm. long, the females 35 to 55 mm., three-fifths of which is formed by the anterior filamentous portion (Blanchard). Diagnosis of infection is made by finding the ova in the feces. The *eggs* (Fig. 34) are oval in shape, with a relatively thick shell, which is generally stained dark yellowish-brown. At either pole there is a space in the shell, which

[36] Stiles, C. W. "Osler's Modern Medicine." Vol. I, 1907, p. 601.

is occluded by a plug, the outer surface of which projects slightly beyond the shell. Within the shell the yellowish or brownish granular yolk substance is seen. The dimensions of the eggs are 0.050 to 0.056 mm. long and 0.024 mm. wide (Blanchard). Though smaller than many other eggs, they are, nevertheless, of sufficient size to be easily seen with the usual low magnifications.

This parasite was found in 5.2 per cent of U. S. troops (see table, p. 160).

Ascaris Lumbricoides.—*Ascaris lumbricoides,* the ordinary "round-worm" of man, is the largest of the commoner parasitic nematodes. Diagnosis may be made by the discovery of the parasite in the feces or vomitus, or of its ova in the stool. The living worm has a reddish



1 2

FIG. 35.—OVUM OF ASCARIS LUMBRICOIDES (1); THE SAME UNDER HIGH FOCUS SHOWING THE ALBUMINOUS COATING (2). ×460.

or grayish-yellow color. The males vary in length between 15 and 25 cm., and are about 3 mm. thick. The posterior end is conical, and is curved ventrally. Females are 20 to 40 cm. long and about 5 mm. in thickness. Lateral, dorsal, and ventral stripes run longitudinally along the body of the parasite, the first being the most prominent. The ova (Fig. 35, 1 and 2) are elliptical and have a thick, transparent shell, which at times appears laminated. A rough albuminous coating forms the outer surface of the egg, and is usually stained brown with the fecal pigments. The albuminous coating may be lost in some of the eggs. The size of the ova varies between 0.040 to 0.050 by 0.050 to 0.070 mm. (Braun). *Unfertilized ova*[37] (Fig. 36) may be encountered.

[37] Logan, O. T. "The little known atypical (unfertilized) egg of Ascaris lumbricoides." *N. Y. Med. Jour.*, 1907, LXXXVI, 1164.

They are flatter—much less plump than the fertilized specimens—the shell is thinner, and the albuminous coating appears to be less abundant. The yolk is coarsely granular, in contrast to the finely granular appearance in the fertilized eggs.

This parasite was encountered in about 1 per cent of U. S. troops (see table, p. 160).

FIG. 36.—UNFERTILIZED OVUM OF ASCARIS LUMBRICOIDES. ×460.

Toxocara Canis.—*Toxocara canis* (*Ascaris mystax*), the common roundworm of dogs and cats, is rare in man.[38] The adult parasite is much smaller than *Ascaris lumbricoides*; the males are 4 to 6 cm. long and 1 mm. thick, while the females measure 6 to 11 cm. in length and 1.7 in thickness. The maximal length recorded is 20 cm. (Blanchard). The *ova* resemble those of *Ascaris lumbricoides*, but are more spherical, having a diameter of 0.068 to 0.072 mm. (Blanchard).

Trichinella Spiralis.—The adult males of *Trichinella spiralis* are 1.4 to 1.6 mm. long and 0.04 mm. thick; the females are larger, measuring 3 to 4 mm. in length, with an average thickness of 0.06 mm. (Blanchard). They inhabit the small intestine. The feces, obtained by active purgation, are mixed thoroughly with water, placed in a tall cylinder, and after the sediment has settled the fluid is poured off. The sediment is then placed in a dish with dark background; the thickness of the fecal layer should not exceed $^1/_{12}$ inch. The dish is tilted, and any minute, hair-like objects are transferred to a slide and examined microscopically (Stiles). The females may remain in the intestines eight weeks; the males die within a few days.

Infection with *Trichinella spiralis* may be detected through the finding of the embryos in the blood (Fig. 88, p. 352), or cerebrospinal fluid (p. 416); or, at a somewhat later stage, by removal of a small piece of muscle (from the calf, biceps, deltoid), in which the encysted embryos may be found.

During the febrile period of trichiniasis, there is usually a leukocytosis (up to 40,000), with increase in the eosinophiles to as high as 70 per cent, as first shown by T. R. Browne.

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[38] Biesele. "Ueber einen Fall von Ascaris mystax beim Menschen." *München. med. Wchnschr.*, 1911, LVIII, 2391.

DIFFERENTIAL CHARACTERS OF THE EGGS OF THE PRINCIPAL NEMATODES *

Membrane	mammillated			45×60μ	*Ascaris lumbricoides*
	smooth	thick	Egg convex on one side, flat on the other, 23×50μ		*Oxyuris vermicularis*
			Clear plug in each pole, 25×55μ		*Trichuris trichiura*
		clear	2 to 4 or more blastomeres	40×60μ	*Ankylostoma duodenale*
				40×70μ	*Necator americanus*
			Embryo developed (egg rare in feces; free embryos found)	32×54μ	*Strongyloides stercoralis*

* After M. Neveu-Lemaire.

Trematodes

Trematodes [39] or flukeworms are fortunately rare in the United States, the cases reported being largely importations from Asia and Africa. Of those whose presence in the body may be determined by finding the ova in the feces, the following are among the more important. The parasites themselves are rarely seen during the life of the host.

Opisthorchis Sinensis.—*Opisthorchis sinensis,* a liverfluke, deposits dark brown, oval eggs with sharply defined operculum or cap. They gain access to the bowel by way of the biliary passages. The ova measure [40] 0.015 to 0.017 by 0.027 to 0.030 mm.

Fasciola Hepatica.—*Fasciola hepatica,* also a liverfluke, is common in many domestic animals (chiefly herbivora), though rare in man. The eggs are oval, yellowish-brown, with distinct operculum, and measure 0.130 to 0.145 by 0.070 to 0.090 mm. They contain no embryo when oviposited (Fig. 40).

Schistosoma mansoni, the causative agent in intestinal bilharziasis (venous distomatosis), inhabits the branches of the portal vein of man, particularly the mesenteric veins. The sharp-spined ova pierce the wall

[39] Stiles, C. W. "Illustrated key to the trematode parasites of man." *Bull. No. 17,* U. S. Pub. Health & Mar. Hosp. Serv., Wash., 1904. (Illustrations and full descriptions of parasites and ova are given, together with brief clinical notes, keys to the ova, etc.)

Ova of the commoner trematodes, drawn to scale, are shown in Fig. 40, p. 187.

[40] The measurements of all trematode ova, unless otherwise indicated, are taken from Stiles (*loc cit.*) and are the extremes reported by him from the literature or his own observations.

of the vessel, and thus it happens that they may be found in the feces. The eggs (Fig. 38) are oval or spindle-shaped and measure 0.060 to 70 by 0.112 to 0.162 mm. (Castellani and Chalmers). The shell is clear, usually brown in color, and is provided with a sharp spine. The latter is situated laterally (subterminally) near one pole of the ovum. The ovum contains a ciliated embryo or miracidium. At times the latter may be seen swimming free in the preparation. The stool contains blood practically without exception.

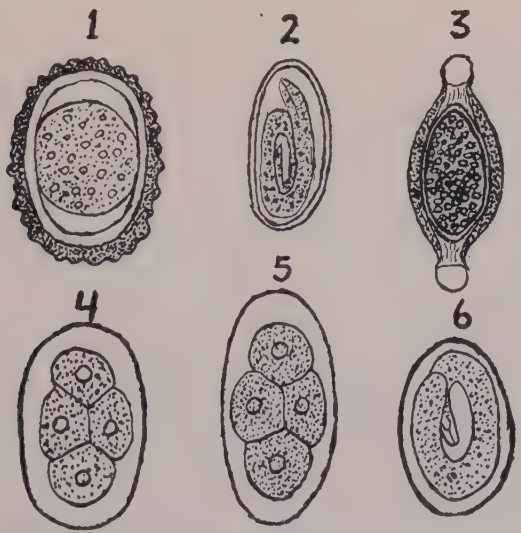


FIG. 37.—OVA OF THE PRINCIPAL NEMATODES PARASITIC IN MAN. Drawn to Scale. ×400. 1, *Ascaris lumbricoides;* 2, *Oxyuris vermicularis;* 3, *Trichuris trichiura;* 4, *Ankylostoma duodenale;* 5, *Necator americanus;* 6, *Strongyloides stercoralis* (after Neveu-Lemaire).

NOTE.—Ova of *Strongyloides stercoralis* are extremely rare in the feces; the embryos only are usually found.

Schistosoma Japonicum.—*Schistosoma japonicum* is endemic in Japan and in certain parts of China, and has been reported from the Philippines. It inhabits the portal and mesenteric veins chiefly; the bladder is apparently unaffected. Infection of the lung (ova in sputum) is rare. The ova (Fig. 39) measure 0.060 to 0.090 by 0.045 to 0.055 mm. (Neveu-Lemaire), are yellowish, double-contoured, without operculum, and have a small, lateral spine, which may be invisible when not seen in profile. Each contains a fully developed miracidium.

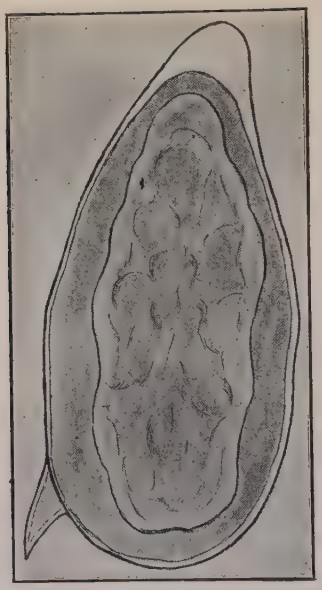


FIG. 38.

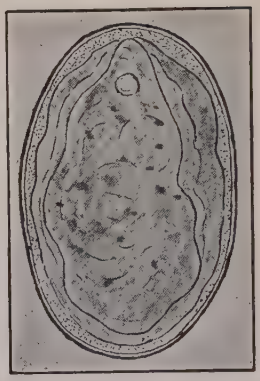


FIG. 39.

FIG. 38.—OVUM OF SCHISTOSOMA MANSONI. From a specimen preserved with formalin. ×460.

FIG. 39.—OVUM OF SCHISTOSOMA JAPONICUM. From a specimen preserved with formalin, obtained through the kindness of Dr. O. T. Logan. ×460.

DIFFERENTIAL CHARACTERS OF THE EGGS OF THE PRINCIPAL TREMATODES *

An operculum	Ovoid; operculum not projecting		80×140μ	*Fasciola hepatica*
			75×125μ	*Fasciolopsis buski*
			55× 95μ	*Paragonimus ringeri*
			25× 40μ	*Dicrocelium dendriticum*
			15× 26μ	*Heterophyes heterophyes*
	Operculum projecting	Prolongation at pole opposite operculum	17× 30μ	*Clonorchis sinensis*
			15× 26μ	*Clonorchis endemicus*
		No prolongation	11× 31μ	*Opisthorchis felineus*
No operculum	Terminal spine		60×150μ	*Schistosoma hematobium*
	Lateral spine		60×150μ	*Schistosoma mansoni*
	Lateral spine (inconspicuous)		40× 75μ	*Schistosoma japonicum*

* After M. Neveu-Lemaire.

Fasciolopsis Buski.—*Fasciolopsis buski* like *Schistosoma japonicum,* is an intestinal fluke. It is widespread in Asia. The ova measure 0.120 to 0.130 by 0.077 to 0.080 mm. and have a delicate operculum (Fig. 40).

Paragonimus Ringeri.—*Paragonimus ringeri,* the lung-fluke, belongs

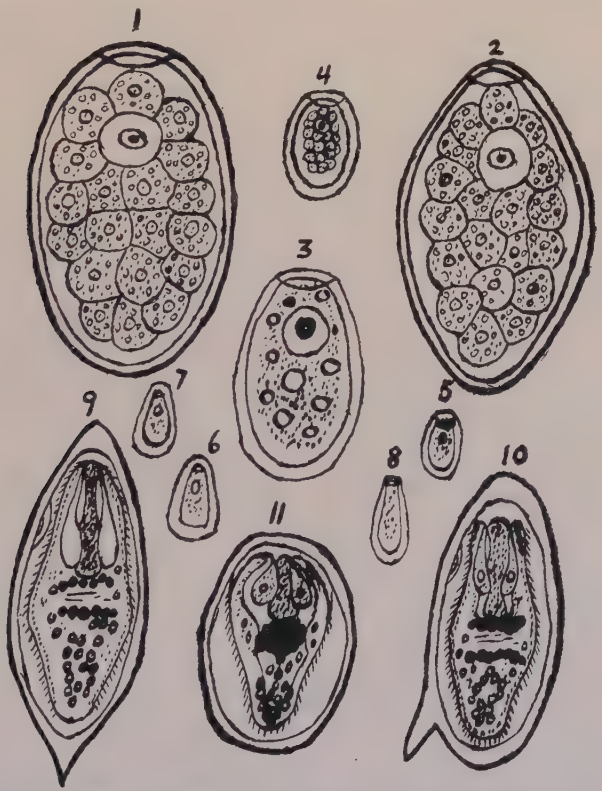


Fig. 40.—Ova of the Principal Trematodes Parasitic in Man. Drawn to scale. ×400. 1, *Fasciola hepatica;* 2, *Fasciolopsis buski;* 3, *Paragonimus ringeri;* 4, *Dicrocoelium dendriticum;* 5, *Heterophyes heterophyes;* 6, *Elonorchis sinensis;* 7, *Clonorchus endemicus;* 8, *Opisthorchis felineus;* 9, *Schistosoma haematobium;* 10. *Schistosoma mansoni;* 11, *Schistosoma japonicum* (after Neveu-Lemaire).

to the class of parasites under consideration. Its ova may appear in the feces through swallowing of the sputa, if they pass the stomach intact. Liver infection has also been recorded. The eggs (Fig. 40 and Fig. 58, p. 223) are oval, 0.068 to 0.118 by 0.048 to 0.060 mm. in size, possess a yellow shell, and are provided with an operculum.

Cestodes

Cestodes [41] or tapeworms include some of the commonest intestinal parasites in the United States. The larger worms usually disclose their presence to the infected individual by segments, which appear in the feces. Microscopic examination of the stools oftentimes reveals ova where infection has not been suspected.

1. **Isaac's Method for Identifying Tapeworm Species.**[42]—For visual-

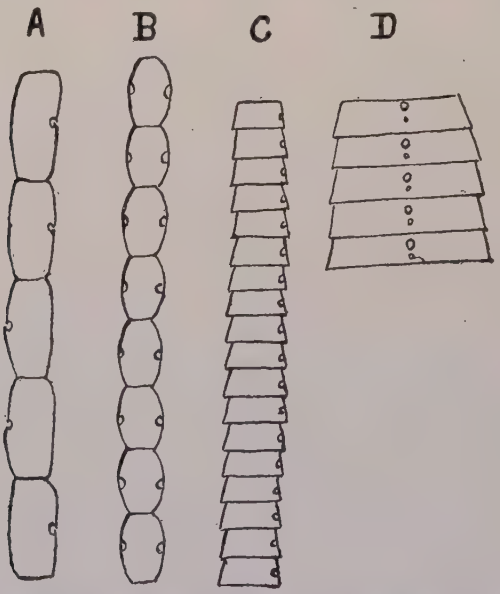


FIG. 41.—DISPOSITION OF THE GENITAL PORES IN DIFFERENT GENERA OF CESTODES. *A, tenia; B, dipylidium; C, hymenolepis* and *davainea; D, dibothriocephalus* (after Neveu-Lemaire).

ization of the uterus in the proglottids of the tapeworm for purposes of identification of species, or for demonstration, the organ may be injected with India ink. A small hypodermic syringe (1 to 2 c.c.), fitted with a fine needle, is filled with India ink, and the point inserted into the

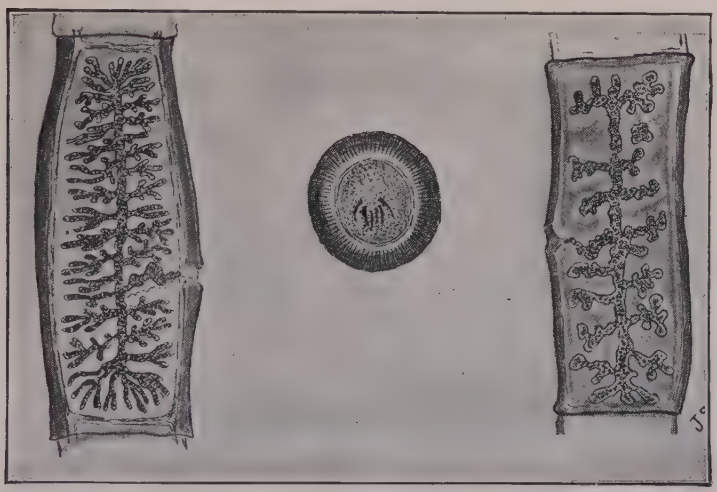
[41] Stiles, C. W. "Illustrated key to the cestode parasites of man." *Bull. No. 25,* Hyg. Lab., U. S. Pub. Health & Mar. Hosp. Serv., Wash., 1906.

[42] An unpublished method of Dr. Raphael Isaacs, to whom the author is indebted for permission to include it here.

Ova of the commoner cestodes, drawn to scale, are shown in Fig. 48, p. 195.

substance of the fresh proglottid near the genital pore on the side. A match stick or wooden applicator may be used to keep the segment from slipping. The ink flows readily into the uterus and outlines the diverticulae, graphically differentiating the sparsely branched organ of *T. solium,* from the more abundant ramifications in *T. saginata.* Pressure between two slides makes examination easier. The preparation may be made permanent by fixation in 10 per cent formaldehyd, dehydration in alcohol, and, after clearing in xylol or carbol-xylol, mounting in balsam. The method is applicable to other species of cestode.

Tenia Saginata.—*Tenia saginata,* the beef tapeworm, is a large para-



(1) (2) (3)

Fig. 42.—1, Gravid Proglottis of Tenia Saginata (×4); 2, Ovum of Tenia Saginata (×460); 3, Gravid Proglottis of Tenia Solium (×4).

site, measuring 4 to 10, even 36 m. in length when fully developed. From the mature, gravid segments (the segments are hermaphroditic), *ova* (Fig. 42, 2) are deposited in the feces. They are round or oval, and measure 0.030 to 0.040 by 0.020 to 0.030 mm. (Neveu-Lemaire). The shell is rather thick, radially striated, and light brown in color. Within it three pairs of hooklets may be visible; to see them it is necessary to focus carefully, as it does not happen often that all are in the same plane. The mature *segments* or *proglottids* (Fig. 42, 1) are those usually seen in the feces. They are 16 to 20 mm. long and 4 to 7 mm. broad, and are characterized by the presence of a uterus with central stem, from

each side of which 25 to 30 lateral branches are given off; these lateral branches are themselves subdivided into numerous smaller branches. The gross structure of the uterus may be determined by flattening the segment between two glass slides and holding it to the light. The uterus then stands out in fairly sharp relief. Each segment is provided with a genital pore which is found at one side; the pores alternate very irregularly from side to side. The head of the parasite is cuboidal, 1.5 to 2.0 mm. thick. It is unarmed.

Note.—From the ova alone it is impossible to distinguish between *Tenia saginata* and *Tenia solium* (q. v.). The ova of *Tenia saginata* are quite innocuous to man, since the intermediary stage of the parasite, *Cysticercus bovis,* to which they give rise, develops practically only in beef—at all events, not in man. With *Tenia solium* the case is quite different. While the hog is the usual host of the *Cysticercus cellulosæ,* the latter may also occur in man, either from the introduction of the ova or of the mature, gravid segments into the stomach. Obviously, then, it is very important to handle all intestinal discharges containing ova like those described above with extreme care, until the presence of *Tenia solium* is definitely excluded. For similar reasons, the patient's excreta should be thoroughly disinfected, preferably by burning.

T. saginata was encountered only twice among 2,876 U. S. troops (p. 160).

Tenia Solium.—*Tenia solium,* the pork tapeworm, resembles *Tenia saginata* in many respects. The fully grown parasite is 2 to 3 m. long. The *ova* are round or oval, 0.031 to 0.036 mm. in diameter (Braun), with brown shell, radially striated. The oncosphere is about 0.020 mm. in diameter, and possesses six hooklets. The egg is indistinguishable from that of *Tenia saginata* (Fig. 42, 2) microscopically. The mature *segments* (Fig. 42, 3), which are often seen in the feces, are 10 to 12 mm. long and 5 to 6 mm. broad (Braun). They differ from the segments of *Tenia saginata* in that the uterus has only 7 to 10 lateral branches extending to either side, and they do not tend to rebranch. The rostellum of *Tenia solium* is characterized by a double crown of 22 to 32 hooklets, large and small alternating. The head of *Tenia saginata,* on the other hand, is unarmed.

Dibothriocephalus Latus.—*Dibothriocephalus latus,* the fish tapeworm, is the third large cestode frequently parasitic in man. The mature parasite may measure 9 m. in length. The *ova* (Fig. 43) have a rather thin, clear, white or brownish shell, with a small operculum or cap. The

DIFFERENTIAL CHARACTERS OF TENIA SAGINATA AND TENIA SOLIUM *

	Tenia solium	*Tenia saginata*
Scolex	Globular, less than 1 mm.	Piriform, more than 1 mm.
	Rostellum and 2 crowns of hooklets	Neither rostellum nor hooklets
	Suckers rounded	Suckers elliptical
Mature segments	Length 10 to 12 mm.	Length 15 to 20 mm.
	Ramifications of uterus coarse, less numerous (5 to 10), dendritic	Ramifications of uterus fine, more numerous (15 to 20), dichotomous
Genital pores	Irregular alternation, at times regular	Alternation much more irregular
Number of segments	700 to 1000	1,200 to 2,000
Total length	2 to 8 meters	4 to 12 meters
Segments expelled	By fragments with the feces	Isolated, apart from defecation
Ova	Spherical, 31 to 36 micra in diameter	Ovoid, 30 to 40 by 20 to 30 micra
Larval stage and intermediate host	*Cysticerus cellulosæ*, in all the tissues of the hog, at times in man. Easy to discover	*Cysticercus bovis*, in the intermuscular accumulations of fat of beef. Difficult to detect.
Infection	Eating infested pork undercooked	Eating infested beef undercooked
Frequence	Very rare in the United States	Fairly common in the United States

* After Neveu-Lemaire, M. *Précis de Parasitologie humaine*, (5th ed.), 1921, p. 239.

latter stands out particularly well after treatment with glycerin or dilute sulphuric acid (Blanchard). The eggs are elliptical, and present granular contents. They measure 0.068 to 0.070 mm. by 0.044 to 0.045 mm. (Blanchard). The posterior segments or proglottids (Fig. 44) may be found in the feces, and at times are devoid of ova. Unlike the two preceding parasites, the majority of the segments are broader than they are long, though the reverse may be observed in the posterior segments. The dark brown, rosette-shaped uterus, placed near the center of the proglottids, distinguishes the parasite. The head, which is almond-shaped, is 2 to 3 mm. long and is unarmed. Hooklets are lacking also in the ova.

FIG. 43.—OVUM OF DIBOTHRIOCEPHALUS LATUS. ×460.

The parasite was found only once among 2,876 U. S. troops (p. 160).

Hymenolepis Nana.[43]—*Hymenolepis nana,* the dwarf tapeworm, is a very common parasite, especially in children. Because of its small size, infection with this parasite is seldom diagnosed by the finding of segments in the feces. The fully developed parasite is 10 to 45 mm. long and from 0.5 to 0.9 mm. thick. The head is round, 0.25 to 0.30 mm. in thickness, and presents a simple crown of 24 to 30 hooklets. The *ova* are spherical or oval (Fig. 45). The shell is clear and transparent, at times having a light brownish or yellowish tint. It consists of two distinct membranes separated by an intervening space, which contains a transparent substance, more or less finely granular. At two opposite

Fig. 44.

Fig. 45.

Fig. 44.—Gravid Proglottis of Dibothriocephalus Latus. ×4.
Fig. 45.—Ovum of Hymenolepis Nana. ×460.

points, usually corresponding to the poles of the egg, there is a small, mammillated projection, often not apparent. To each of these is attached a number of clear hyalin fibers, which pass out through the intermediate substance toward the outer membrane. It frequently happens that the intermediate substance shrinks or retracts from the outer or inner membrane or from both, resulting in the appearance of a third membrane between the two; in reality none exists. The outer membrane is very thin, less than 0.001 mm. The inner membrane is of about the same thickness, and closely invests the oncosphere, which presents three pairs

[43] Ransom, B. H. "An account of the tapeworms of the genus Hymenolepis parasitic in man, etc." *Bull. No. 18,* Hyg. Lab., U. S. Pub. Health & Mar. Hosp. Serv., Wash., 1904. (A full description of the parasites, with the clinical aspects of infection.)

of hooks, usually directed toward one pole (Ransom). The outer dimension of the egg varies between 0.036 and 0.056 mm. long and 0.032-0.042 mm. broad.

This tapeworm was found in about 0.5 per cent of U. S. troops (p. 160).

Hymenolepis Diminuta.—*Hymenolepis diminuta,* commonly found in rats, is occasionally parasitic in man. The parasite is small, being 1 to 6 cm. long and 2.5 to 4 mm. wide. The head, which is unarmed, is 0.2 to 0.6 mm. wide. The *ova* (Fig. 46) resemble those of *Hymenolepis nana.* They are round or nearly so, and have two membranes. The outer membrane may be radially striated. The intervening space between the two membranes is granular. The diameter of the eggs varies between 0.054 and 0.086 mm. (Ransom).

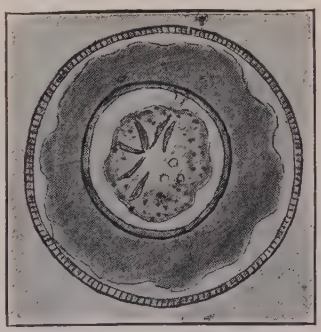


Fig. 46.—Ovum of Hymenolepis Diminuta. ×460.

Davainea Madagascariensis.—*Davainea madagascariensis* (syn.: *Tenia madagascariensis; T. demarariensis*) is a rare tapeworm. The worm is 25 to 30 cm. in length, with a maximum breadth of 1.4 mm. The scolex has four large suckers and a rostellum with ninety hooks. The proglottids number from 500 to 700, being 2 mm. long by 1.4 mm. broad; genital pores are unilateral and near the proximal corner. The ova measure 0.040 mm. and contain an onchosphere (embryo) 0.008 by 0.015 mm.

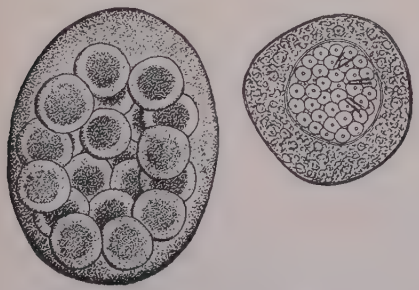


Fig. 47.—Dipylidium Caninum. Showing an egg capsule and a free ovum (After Stiles).

Dipylidium Caninum.—*Dipylidium caninum* is a common parasite in the intestines of dogs and cats; a number of instances of human infection are recorded.[44] The fully developed parasite is 15 to 35 cm. long The head is small and the rostellum club-shaped, with

[44] Lins, J. "Sechs Fälle von Tænia cucumerina beim Menschen." *Wiener klin. Wchnschr.,* 1911, XXIV, 1595.

three to four rows of hooks, about 60 in number. The mature, gravid segments may be seen without difficulty with the unaided eye. They are 8 to 11 mm. long and 1.5 to 3 mm. broad; their color is often reddish. The genital pores are double, and are opposite. The *ova* (Fig. 47) are spherical, and have a diameter of 0.043 to 0.050 mm. The shell is thin. The uterus contains the eggs in capsules, 8 to 20 eggs being contained in each; they may be encountered in the feces in this form (Stiles). Three pairs of hooks are to be seen in the oncosphere.

Differential Characters of the Principal Tapeworm Ova *

No operculum	A single membrane	Thick and opaque	Ovum spherical	31 to 36μ. *Tenia solium*
			Ovum ovoid	35 × 25μ. *T. saginata*
		Thick and transparent		40 to 50μ. *Dipylidium caninum*
	Three transparent membranes			40 × 50μ. *Hymenolepis nana*
				60 to 80μ. *H. diminuta*
An operculum				45×70μ. *Dibothriocephalus latus*

* After Neveu-Lemaire.

Preservation of Gross Specimens of Cestodes and Other Parasites

Stools containing *parasitic ova or embryos* are best preserved by adding commercial formalin (40 per cent) to a thin watery suspension of the material, so that the latter contains about 2 per cent of formalin. The majority of eggs are quite well preserved, though thin-shelled ova, such as those of *Hymenolepis nana,* show considerable distortion. Specimens of feces containing the rhabditiform embryos of *Strongyloides stercoralis* may be kept for several years; the structure may be wanting in some of the embryos, but may remain characteristic in others. It is of interest that the formalin may not arrest the development of the ova of *Ascaris lumbricoides.*[45] The writer has one specimen of feces now more than four years old, preserved with formalin; many of the *Ascaris* ova contain living embryos. The adult parasites may also be preserved in formalin (2 per cent solution).

[45] Morris, R. S. "The viability of parasitic ova in two per cent formalin, with special reference to Ascaris lumbricoides." *Johns Hopkins Hosp. Bull.*, 1911, XXII, 299.

Permanent Preparations of Flatworms

For purposes of study cestode and trematode material may be prepared in several ways.

1. **Isaacs' Method.**—See p. 188.

2. **Method of Mink and Ebeling.**[46]—The fecal material is mixed with physiological salt solution heated to about the body temperature (37° to 40° C.). The worms move about, and the smaller, such as *Hymenolepis nana,* are the more readily seen. With forceps the parasites are transferred to a second dish of clear salt solution, in which they become free of mucus and feces. They are now transferred to one of

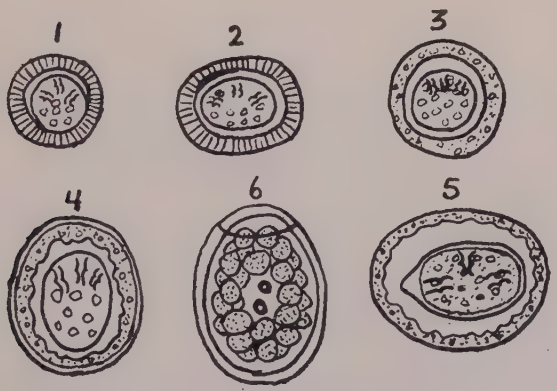


FIG. 48.—OVA OF THE PRINCIPAL CESTODES PARASITIC IN MAN. Drawn to scale. ×400. 1, *Tenia solium;* 2, *Tenia saginata;* 3, *Dipylidium caninum;* 4, *Hymenolepis nana;* 5, *Hymenolepis diminuta;* 6, *Dibothriocephalus latus* (after Neveu-Lemaire).

three solutions for fixation: (1) Alcohol, 50 to 70 per cent, with or without glycerin; (2) Zenker's solution (which consists of bichlorid of mercury, 5.0 gm.; potassium bichromate, 2.5 gm.; sodium sulphate, 1.0 gm.; distilled water to 1,000 c.c.), or (3) a 2 per cent formalin solution. In any of these the material remains 14 to 15 hours. Zenker's fluid causes considerable shrinking and a yellowish discoloration. Formalin is best, since the natural color of the parasite is preserved fairly well with little or no shrinkage. The fixative should be allowed

[46] Mink, O. J., and Ebeling, A. H. "A method for the preparation of flatworms for study." *U. S. Naval Med. Bull.,* 1909, III, 267.

to act not more than fifteen hours, when the parasites are transferred to the following medium:

Syrup (glucose, 48 parts, water, 52 parts)	1,000.0 c.c.
Methyl alcohol	200.0 c.c.
Glycerin	100.0 c.c.
Camphor, q. s. (to keep sterile).	

The specimens may be left in this solution indefinitely, though they are usually sufficiently cleared in 4 to 5 hours. The material is now placed on a slide in glycerin jelly. After the latter has hardened (24 hours or more), the cover glass is sealed with cement.

3. **Boggs' Method.**[47]—The worm is washed free of feces, and is placed in water or salt solution, in which it is allowed to die, so that it may be fixed while relaxed. It is then placed in a solution of 20 per cent glycerin in 80 per cent alcohol, which both fixes and clears the specimen. It is allowed to remain in this fluid in a partially covered dish until the alcohol is entirely evaporated. The specimen is then clear. It is transferred to a glass slide, and the excess of glycerin is removed by blotting paper. Glycerin jelly is then placed on the specimen, which is covered with a cover glass. In 24 to 48 hours, after the jelly has solidified, the preparation is sealed with microscopical cement. To prevent curling of the specimen, it may be spread on a piece of heavy filter paper before immersing it in the glycerin-alcohol solution; it may be necessary to put a light weight on the cover slip until the jelly hardens.

Glycerin jelly is prepared as follows:

Gelatin (gold mark)	14.0 gm.
Distilled water (boiling)	120.0 c.c.
Dissolve in the hot water and add—	
Glycerin	120.0 c.c.

Cool to 50° C. and add the whites of two eggs. Heat gently without stirring. Strain the mixture through a fine-meshed wire sieve, and filter through cotton while still warm. Add water to make the volume 240 c.c. and 1 c.c. of pure carbolic acid as a preservative. The jelly solidifies on cooling. For use, melt it by immersing the flask containing it in hot water.

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[47] Boggs, T. R. Personal communication, and in Emerson, C. P. "Clinical Diagnosis." 3d. ed. Philadelphia and London, 1911, p. 444.

4. **Creosote Method.**—The material is placed in 70 per cent alcohol, and then in 95 per cent alcohol, for 15 to 30 minutes. It is then transferred to Beechwood creosote, in which it remains until the tissue is

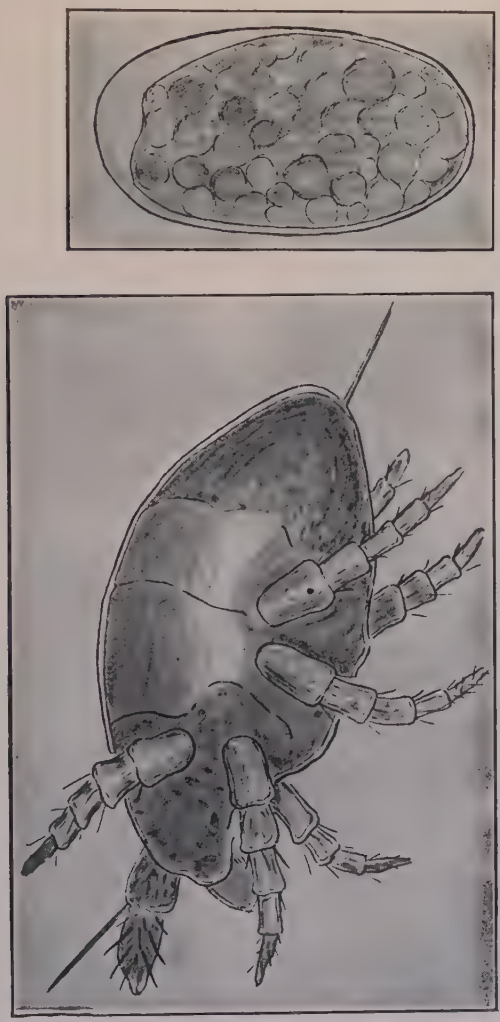

FIG. 49.—TYROGLYPHUS SIRO, the CHEESE-MITE and OVUM. ×460.

cleared. The time required varies with the size of the specimen and with the degree to which water was withdrawn by the alcohol. Finally, the specimen is mounted in balsam.

Previous to mounting, the material may be injected by Isaacs' method (p. 188).

Accidental contaminations through food or drink may account for some of the *ova* found in the feces. As an example, the ova of the *Tyroglyphus siro* (Fig. 49) may be cited. This is the common cheese-mite, which may also be found in flour and other articles of diet. The mites may be found in the stool in addition to the ova. Measurements of the ova may serve to differentiate them from those of intestinal parasites. In cases of doubt, it is advisable for physicians to submit the material to a zoölogist for determination. Such examinations are made at the Hygienic Laboratory, U. S. Public Health Service, Washington, D. C.

CHAPTER IV

THE SPUTUM

In the strict sense of the word, sputum refers to the expectorated material which arises in the respiratory passages between the lung alveoli and the larynx.

Sputum for Examination.—To obtain sputum for examination the patient should be told to discard the nasal and pharyngeal discharges. He should be instructed as to the proper receptacle. In case the physician does not supply a sputum box or cup, the patient may conveniently use a *wide-mouthed* bottle, which has been thoroughly cleansed and sterilized by boiling. In cases where a sputum examination is urgently indicated but no sputum is expectorated, expectorants, such as ammonium chlorid, may be given. Hausmann [1] advises that the fasting stomach be washed out in the early morning with a view to obtaining bronchial mucus; he reports valuable findings with this method. With children it is not infrequently necessary to wash out the stomach, examine the feces, or place the finger, covered with gauze, in the child's throat after a coughing spell, and mop out the sputum.

The importance of repeated examinations of the sputa cannot be overemphasized. Particularly when looking for the tubercle bacillus, if the physical signs or history are even suggestive, examinations should be continued. A single negative result means nothing.[2]

Amount.—The quantity of the sputum expectorated in twenty-four hours varies greatly in disease. An approximate estimate of the amount is usually sufficient.

Reaction.—The reaction of fresh sputum is usually alkaline. An old specimen or sputum which has stagnated in the body may be acid in reaction.

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[1] Hausmann, T. "Die Frühdiagnose der Lungentuberkulose durch die Mageninhaltsuntersuchung." *Deutsch. Arch. f. klin. Med.*, 1908, XCIV, 595.

[2] Brown, Lawrason. "Certain fundamentals in early diagnosis of pulmonary tuberculosis." *J. A. M. A.*, 1922, LXXVIII, 79.

Character.—Sputum is designated *mucoid, mucopurulent, purulent, serous,* or *bloody,* as the case may be. The terms are self-explanatory. Various combinations are met with. Bloody sputa may assume any of the shades seen in a bruise. In the presence of jaundice, it must be remembered that green sputa do not necessarily indicate a previous pulmonary hemorrhage; the color is in most cases simply a manifestation of the icterus. Bacterial growth may at times account for a green color.

Odor.—The odor of sputa is important chiefly in connection with putrid affections of the bronchi or lungs.

Consistence.—The consistence of the sputum is generally dependent on the quantity expectorated. With large quantities, the consistence is usually thin, though the sputa of croupous pneumonia form a notable exception. Similarly, when the sputum is small in amount, it is usually more or less tenacious.

Air Bubbles.—Most sputa contain air bubbles. The size of the bubbles is said to indicate roughly the caliber of the bronchi from which the expectorated material is derived. More air is contained in sputum from the smaller bronchi than from the large.

Dittrich's Plugs.—Dittrich's plugs are sausage-shaped casts of the bronchi, varying in size up to that of a white bean. Microscopically, they may be seen to contain fat droplets, fatty acid crystals, cell detritus, and bacteria. A few pus cells and occasionally red blood corpuscles or hematoidin in granules or needles may be observed, less commonly flagellates.

Bronchial Casts.—Bronchial casts are observed in some diseases with considerable frequency. Their size is determined by that of the bronchi giving rise to them. Casts are usually branched, and consist mainly of fibrin, in which leukocytes, red blood cells, epithelial cells, etc., may be embedded. If the casts are small, their isolation is facilitated by placing the sputum on a white plate, half of which has been painted black. By teasing the specimen with needles, the casts may be found, provided they are macroscopic in size. The addition of water often renders the teasing easier.

Curschmann's Spirals.—Curschmann's spirals (Fig. 51), occur in various sizes. Only the larger specimens are visible to the unaided eye. The largest spirals measure about 1 mm. in thickness, and are ten to twenty-five times as long. They can only be distinguished as spirals by microscopic examination. They are found in the sputum of patients suffering with bronchial asthma.

Layer Formation.—When abundant, as in the case of sputa from bronchiectatic cavities, for example, distinct layer formation may be observed. Solid particles collect at the bottom, then a fluid portion, with frothy mucus on the surface. Frequently strands of mucus dip down from the upper layer.

GROSS APPEARANCES OF THE SPUTA IN DISEASE

There are some respiratory diseases in which the gross or macroscopic appearance of the sputa is more or less characteristic, while with others the inspection of the specimen is of little or no assistance in arriving at a diagnosis.

Pneumonia.—In acute lobar pneumonia the sputum is usually quite characteristic. It is extremely tenacious, so that one may invert the cup in most cases without losing a particle of sputum. The color of the sputum is also important in this disease. It may be bloody from the onset, but is usually mucoid, becoming blood-tinged after the first twenty-four hours, or so. As the disease progresses, the sputum becomes rusty from the presence of the altered blood. Still later in the disease, the sputum may resemble prune juice. When the disease is complicated with jaundice, a yellowish or greenish color may be noted in the sputum. Occasionally the sputum remains blood-streaked throughout.

Tuberculosis.—There is no characteristic macroscopic appearance of the sputum in pulmonary tuberculosis. In a suspected case, *any* sputum which the patient expectorates should be carefully examined. At times, tubercle bacilli are found in mucoid sputum, though as a rule purulent particles are present, and the organisms are more apt to be found in these than in mucoid masses. In advanced cases, the sputum is often expectorated in coin-shaped or round purulent masses, the so-called nummular sputum. The specimen is often blood-streaked, and at times there is a larger hemorrhage. The examination for tubercle bacilli is greatly facilitated by the use of the plate method (see p. 204); particles of elastic tissue, if found, should be selected for staining.

Influenza.—The sputum is often abundant, thin and watery, and contains purulent particles. Pfeiffer considered sputum containing nummular, purulent masses of a yellowish-green color as distinctive of the disease. At times, the sputum has a dark red color from the presence of blood.

Whooping Cough.—In the catarrhal stage, there is usually no sputum. In the paroxysmal stage, the sputum is very tenacious and mucoid. The

Bacillus pertussis of Bordet and Gengou is present, especially during the first two weeks of the paroxysmal stage.

Bronchial Asthma.—"The sputum is distinctive. Early in the attack it is brought up with difficulty and consists of small round masses, gelatinous, like sago balls in a thin mucus, the so-called 'perles' of Laennec" (Osler). In other cases, the sputum is more abundant, at times frothy, and occasionally blood-streaked. Examination on a glass plate reveals the characteristic Curschmann's spirals, together with eosinophiles and Charcot-Leyden crystals. The sputum may amount to 100 to 200 c.c. in a day.

Bronchiectasis.—With extensive saccular dilatations the patient coughs up large amounts of sputum once or twice a day. The quantity varies, usually between 100 c.c. and 600 c.c., though as much as a liter may be expectorated in twenty-four hours. Often the gross appearance is quite characteristic. The color is gray or grayish-brown; occasionally bright red blood is found. The odor of the sputum may be fetid. It separates into three layers when placed in a conical sedimenting glass; the top layer is frothy and brownish, from which strands of mucus may extend downward into the rather turbid, fluid middle layer, while at the bottom there is a thick grayish or brownish sediment.

Fibrinous Bronchitis.—Casts of the bronchi are expectorated, during paroxysms of cough.

Spirochetal Bronchitis.—(*a*) *Acute* cases: The expectoration is scanty, mucopurulent, very seldom containing traces of blood. (*b*) *Subacute* cases: There is expectoration of pink, jellylike mucus; true hemoptysis may occur. (*c*) *Chronic* cases: The expectoration is not very abundant, and may be mucopurulent; in many cases for periods of two or three days or longer, the expectoration contains blood. Sometimes attacks of genuine hemoptysis occur, the blood amounting to one or two teaspoonsful, while occasionally a larger hemorrhage occurs.

Abscess of the Lung.—The quantity of sputum is variable, depending on the size and number of the abscesses and the freedom of communication with the bronchial tree. The sputum is purulent, at times blood-stained, and the odor offensive in many cases, though not the putrid odor of pulmonary gangrene or putrid bronchitis. Occasionally, macroscopic bits of necrotic lung tissue are expectorated. Examination with the plate method reveals yellow elastic tissue.

Gangrene of the Lung.—The sputum is generally large in amount, and when placed in a conical glass, it separates into three layers, as in

the case of bronchiectasis. There is usually a heavy, greenish-brown sediment, an intervening thin liquid, which may have a greenish or brownish tint, and on top a thick, frothy layer (Osler). As in the case of lung abscess, bits of necrotic tissue, varying in size, in which elastic tissue can be demonstrated, may be expectorated.

Putrid Bronchitis.—The sputum has a fetid odor, is large in amount, as a rule, has a grayish color and separates into three layers, like the sputa of bronchiectasis and pulmonary gangrene. In the sediment, Dittrich's plugs may be found.

Lung Infarct.—The sputum is bloody, the color often being dark red. It is associated with disease of the heart in the great majority of instances. The amount of blood is variable. The sputum is usually tenacious.

Empyema Rupturing into the Bronchial Tree.—The material expectorated is abundant and is purulent. Yellow elastic tissue and bits of necrotic tissue are lacking. At times, *exploratory puncture* yields a few drops or a few c.c. of pus in cases of suspected empyema, the result of the puncture apparently confirming the clinical diagnosis. In such cases, microscopic examination of the *fresh* pus should always be carried out; the finding of *dust cells* or of *yellow elastic tissue* shows conclusively that the pus came from the lumen of a bronchus or from an abscess, *not* from the pleura.

Liver Abscess Rupturing into the Bronchial Tree.—The patient expectorates purulent matter having the appearance of anchovy sauce, the color being reddish-brown, due to red cells and altered hemoglobin. In case the liver abscess is amebic (and this is the usual etiology), motile forms of *Entameba dysenteriae* may be found in the fresh sputum.

Edema of the Lungs.—There is a thin, frothy, albuminous expectoration, often with a pinkish color from the presence of small amounts of blood. The *albuminous expectoration,* which follows thoracentesis in rare instances, is due to a pulmonary edema.

Pulmonary Streptothromycosis (*Pulmonary Nocardiasis; Pseudotuberculosis*).—The sputum is mucopurulent and contains blood at times. Since the *Nocardia* are often present in the sputum as acid-fast rods which may or may not appear in branched form, the differentiation from tuberculosis may be difficult; cultures of the sputum may be necessary. Other species of *Nocardia* found in the sputum are not acid-fast. Occasionally, small, white granules, consisting of masses of the fungus, are present (Castellani and Chalmers).

Paragonimiasis.—The sputum is purulent and in most cases blood-stained. The ova of the parasite, *Paragonimus ringeri,* are best seen by the addition of a little 0.1 per cent solution of sulphuric acid (Castellani and Chalmers).

Pneumoconiosis.—The sputum is mucopurulent, at times abundant, and the color dark, even black (anthracosis). Dwellers in smoky atmospheres frequently expectorate dark, mucoid sputum in the morning.

The sputa of *acute* and *chronic bronchitis*, of *broncho-pneumonia*, as well as of other respiratory diseases less commonly encountered, are not distinctive.

MICROSCOPIC EXAMINATION

Examination of the Fresh Sputum.—Examination of the fresh sputum is greatly neglected. It is as important in the routine study of sputa as the various staining methods, and may furnish information which can be gained in no other way.

For the examination of the fresh specimen two *glass plates* are required, one about the size of the stage of the microscope or a little smaller, the other of the same length or even a bit longer, but about one inch narrower. Old photographic plates, when cleansed, answer the purpose very well; they may be cut into any size desired. In place of the second plate, a glass slide (3×1 in.) may be used. For handling the sputum steel hat-pins are useful. They are inexpensive, and are easily sterilized by heating in the flame of the Bunsen burner.

Some of the sputum to be examined is transferred to the larger plate by means of the hat-pins. It should be so placed on the plate that, when spread out, all parts of the specimen will be accessible for microscopic examination. After sterilizing the hat-pins [3] the second plate or the glass slide is placed over the sputum, which is spread out in a thin layer. Examination is made with the low power objective, for the thickness of the upper plate is usually greater than the working distance of the higher power dry objective. Furthermore, higher magnification is often unnecessary. The magnification may be increased, however, by selecting a strong ocular or by drawing out the tube of the microscope.

After spreading the sputum between the two plates, it is examined macroscopically on a dark background, and any small, opaque masses are noted and singled out for microscopic inspection. Examination then determines whether the particle should be transferred to a slide and

[3] All sputa should be considered as infectious material, and handled accordingly.

studied under a cover glass with higher magnification, or used for staining, or for both purposes. Curschmann's spirals, necrotic tissue fragments, etc., are looked for in this way. The macroscopic examination should always be made, for a great deal of time may be wasted, to say nothing of overlooking important findings, if the microscopic examination is performed aimlessly. There are, of course, specimens in which macroscopic examination reveals nothing, where microscopic study of the preparation is fruitful. But in the majority of instances the naked eye inspection of the thinly spread specimen is an important adjuvant to microscopic examination.

Yellow Elastic Tissue.

This *tissue* may be found in the sputum, when there is ulceration of the lungs or bronchi with necrosis:

1. Pulmonary *tuberculosis.*
2. Lung *abscess.*
3. *Gangrene* of the lung.
4. *Bronchiectasis* occasionally.

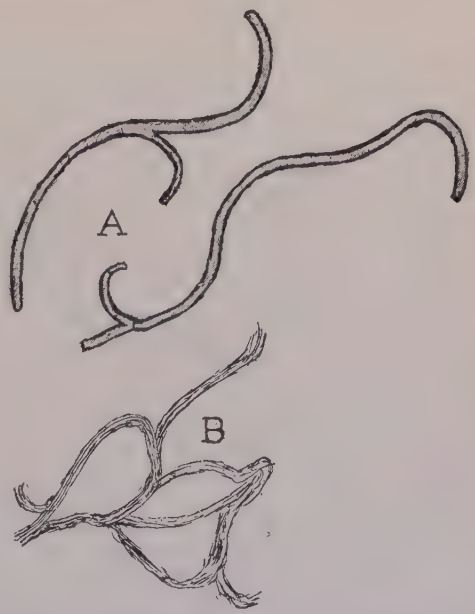

FIG. 50.—SPUTUM. Yellow elastic tissue. *A*, single fibrils, highly magnified; *B*, alveolar elastic tissue, lower power.

Elastic tissue fibers (Fig. 50) are most easily found by means of the glass plate method. If present, they are found in yellowish, opaque masses of necrotic tissue about the size of a pinhead or thereabouts. The fibers may present roughly the outline of one or more alveoli (alveolar elastic tissue), single fibers or several long fibers forming a network may be seen (bronchial), or there may be sheets of elastic tissue (arterial). Often only isolated fibers are met with. The yellow elastic fibers are readily seen, and are characterized by (1) their uniform diameter, (2) sharp outline, (3) great refractivity, (4) curling ends, (5) a tendency to branch, and

(6) by the fact that pressure on the slide produces no varicosities or thickenings in the fibers.

Fatty acid crystals, which may be mistaken for elastic tissue, usually present varicosities after pressure, and are, furthermore, unlike elastic tissue in that they are soluble in ether or KOH. Again, warming the preparation transforms fatty acid needles into droplets. In contrast to the wavy elastic tissue fibers, fatty acid crystals usually present a single curve.

Curschmann's Spirals.—Curschmann's spirals (Fig. 51), often visible macroscopically but never recognizable as such, stand out with distinct-

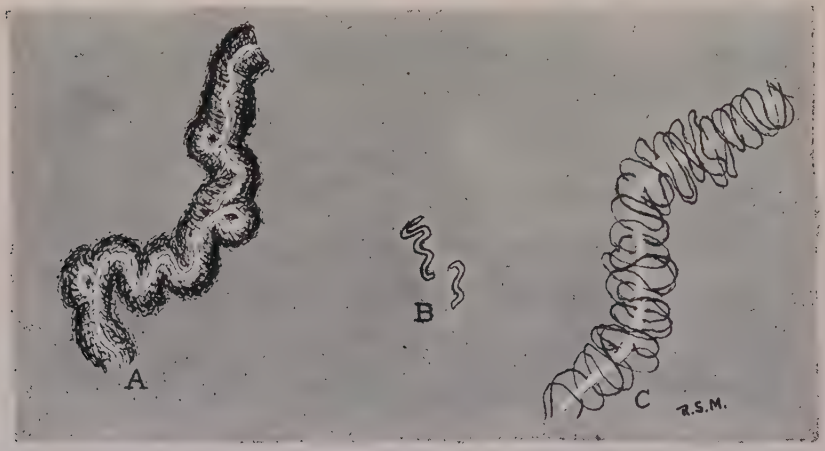

FIG. 51.—SPUTUM. Curschmann's spirals. *A*, spiral with tightly wound mucus; *B*, spirals, natural size (after Sahli); *C*, spiral with loosely wound threads of mucus (spirals are also found without the refractive central filament, and the filament may be found free in the sputum).

ness on microscopic examination. They occur in two forms; in the one there is seen a twisted spiral consisting of delicate, thread-like filaments of mucus, in the turns of which eosinophilic leukocytes, pus cells, epithelial cells, Charcot-Leyden crystals, etc., are caught; in the other form there is a highly refractive central filament, about which the mantle of mucus containing eosinophiles, etc., is twisted. The spirals are subject to much variation in size. Isolated central filaments may be found. The thickness of the filaments differs in different spirals, but in a given specimen it is quite uniform. The finest filaments are extremely minute, while the largest may be twice as thick as a red blood corpuscle.

Fairly satisfactory permanent preparations of spirals may be had by mounting them in glycerin jelly and sealing the specimen with cement after the jelly has hardened.

Dust cells, "heart failure" cells, Charcot-Leyden crystals, and other objects may be seen on examining the sputum on the glass plate. The Charcot-Leyden crystals may be so small as to escape detection. It may, therefore, be advantageous to use a *higher magnification* in their study. For this a selected particle of sputum is transferred to a glass slide and pressed out under a cover glass.

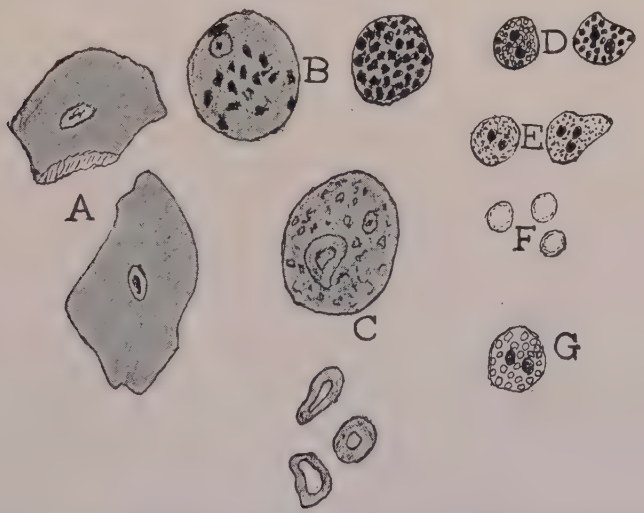

Fig. 52.—Cells in Sputum. *A*, squamous cells from the pharynx; *B*, alveolar epithelial cells, containing coal dust ("heart failure" cells are the same, except that the altered hemoglobin has a yellow or golden brown color); *C*, myelin droplets, intra- and extra-cellular; *D*, pus cells containing coal dust; *E*, pus cells; *F*, red blood cells; *G*, eosinophile cell.

Alveolar epithelial cells (Fig. 52, *B, C*), derived from the lung alveoli, are constantly present in the sputum. Their shape varies greatly, since they are possessed of ameboid motion—a fact which is readily demonstrable by examining a perfectly fresh specimen on a warm stage. They are relatively large cells, but are not of uniform size. The nucleus is large and oval. The protoplasm of the alveolar cells is rather coarsely granular, but soon undergoes degeneration, as a result of which fat droplets and myelin granules make their appearance in it.

The *fat droplets* in alveolar cells are, like similar droplets elsewhere, of all sizes, usually round, refractive, and slightly greenish, especially

the larger drops. Their nature is determined by adding a drop of alcoholic solution of Sudan III or Scharlach R to the specimen, by means of which fat is stained orange or orange-red.

Myelin degeneration (Fig. 52, *C*) of the alveolar cells gives rise to the macroscopic masses in sputa resembling boiled sago. The myelin droplets may be large or small, and, unlike fat droplets, they are often quite irregular in contour. At times the center of a mass of myelin appears to be thinned. Myelin granules are refractive and have a greenish tint, which is more pronounced than that seen in fat droplets. Myelin is frequently found free in the sputum, probably the result of disintegration or mechanical rupture of the degenerated epithelial cells. It does not take the fat stains.

Dust cells (Fig. 52, *B*) are found in the sputum of all who inhale coal dust. They are alveolar epithelial cells, which have phagocyted the minute particles of coal dust, which constantly are inspired in a smoky atmosphere. The dust appears as dark, brownish-black spots in the cell, which may be so heavily laden that nucleus and protoplasm are entirely obscured. Dust cells are easily distinguished in examining sputum by the plate method. When they are numerous, the sputum is stained more or less diffusely black. At times, leukocytes engulf the coal dust (Fig. 52, *D*).

"Heart-failure cells" are alveolar epithelial cells which have taken up blood pigment. The name is a misnomer, for they may appear in the sputum after a pulmonary or bronchial hemorrhage from any cause whatever. The pigment, which is hematoidin, appears as light, golden-yellow granules, which can scarcely be confused with coal dust.

Red blood corpuscles (Fig. 52, *F*) are often seen in a state of preservation. If the hemorrhage is an old one, however, the cells disintegrate, and only hematoidin in amorphous masses—usually in heart-failure cells—or in needles will be discovered.

Blood in the sputum (*hemoptysis*) may be observed in:

1. *Young, healthy adults.*
2. Pulmonary *tuberculosis* (in 60 to 80 per cent of all cases).
3. *Pneumonia.*
4. *Abscess* of the lung.
5. *Gangrene* of the lung.
6. *Foreign bodies* in the lung.
7. *Bronchiectasis.*

8. *Spirochetal bronchitis.*
9. Pulmonary *infarct.*
10. *Gassing* in warfare (immediate and late—as long as two years after).
11. Chronic *valvular disease,* especially mitral stenosis.
12. *Ulcerations* of the respiratory passages.
13. *Aneurism* of the thoracic aorta (slight bleeding for months or a sudden large hemorrhage).
14. *Hypertension* (at times profuse).
15. *Malignant fevers.*
16. *Purpuras, acute leukemias,* etc., at times.
17. *Vicarious (menstrual) hemorrhage.*
18. *Parasitic* or *endemic hemoptysis* (due to *Paragonimus ringeri*).
19. *Bronchomoniliasis.*
20. *Penetrating wounds* of the chest (gun-shot, stab, etc.).

Pus cells (Fig. 52, *E*), polynuclear neutrophilic leukocytes, are always present in the sputa microscopically. The polymorphous nucleus in a cell about 12 micra in diameter with finely granular cytoplasm is characteristic. Occasionally fat droplets are contained in the cells, or they may take up foreign particles in the air passages. In a fresh specimen active ameboid movements may be observed in these cells; pseudopodia are protruded, and the granules of the protoplasm are actively motile.

Eosinophilic leukocytes (Fig. 52, *G*), of the same size as pus cells and having a polymorphous nucleus, are distinguished by their protoplasmic granules. The latter are coarser than the neutrophilic granules, and are highly refractive, glistening bodies. Ameboid motion may also be observed in these cells. Free granules are usually present in the specimen.

Lymphocytes, or cells which are identical morphologically, are present at times in large numbers. The round or oval nucleus with narrow rim of protoplasm and the small size of the cells—7 to 12 micra—together with the non-granular cytoplasm are distinctive.

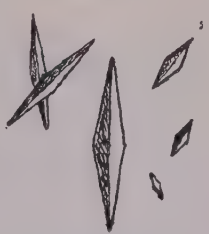

FIG. 53.—SPUTUM. Charcot-Leyden crystals.

Charcot-Leyden crystals (Fig. 53) are usually found in the sputum with large numbers of eosinophile cells. They are formed wherever eosinophile cells disintegrate. In form the crystals are long lozenges. Their edges are clean cut, the points

sharp, and the crystals have a yellowish or greenish tint. They are quite fragile, and the larger crystals may be broken in preparing the specimen. They occur singly or in clusters, and vary greatly in size; the smallest are visible only with the oil-immersion objective, while large crystals are seen without difficulty with low magnification. On cross-section the crystals are hexagonal. Like the eosinophile granules, they may be stained with eosin. They are soluble in mineral salts, alkalies and boiling water. The crystals may be apparently lacking in sputa which contain enormous numbers of eosinophiles. This is particularly apt to be the case when a sputum is first flooded with these cells. If examinations are made from day to day, the crystals are found sooner or later.

Crystals of fatty acid, cholesterin, hematoidin (Fig. 13, p. 85), *triple phosphate* (Fig. 14, p. 88), etc., may be encountered in sputa, especially after stagnation. (For morphology and microchemical reactions see the chapter on the urine or feces.)

MICROÖRGANISMS IN SPUTA

Only the more important microörganisms of the sputum are referred to in the following pages, and in no case are cultural methods described. For this and the finer details of morphology, the reader is referred to works on bacteriology.

Bacillus Tuberculosis

Bacillus tuberculosis (Plate V, *E*) may be found in any kind of sputum, for the gross appearance of the sputum in pulmonary tuberculosis is in no way distinctive; it may, in fact, be anything. If the plate examination shows necrotic tissue (elastic tissue), it should be selected for staining, as the bacilli are generally more numerous in such material. Otherwise purulent particles, when present, are most suitable for examination.

Morphology.—Tubercle bacilli are elongated rods, measuring 2 to 4 micra in length and about 0.2 to 0.4 micron in thickness. The bacilli may stain diffusely; often beaded forms are encountered in the sputum. Branching forms of the organism, resembling somewhat branching, acid-fast species of *Nocardia*, are at times found. The diameter of individual bacilli may not be uniform; ovoid or rounded thickenings may be noted, often staining intensely. Often the bacilli are found in clumps of three or more, but occasionally isolated organisms are found in the sputum.

When only one or two bacilli are found in the stained smear, the result should be confirmed before a positive diagnosis is made.

Preparation of Smear for Examination.—The preparation for examination is made by smearing the selected particle on a glass slide with a hat-pin or other suitable object. Or it may be pressed between two slides, which are then drawn apart, so that the material is smeared in a thin layer on each of them. In the second way preparations of more uniform thickness are obtained, but there is usually some of the material at the edge of the slide, with which the fingers or other objects may become contaminated. (This is referred to not because it is a valid objection to the method, but because the writer has so frequently observed carelessness in this particular point. Still, one who neglects such an obvious source of infection is sure to make other more serious breaks in technic, and has no business examining infectious material, both for his own safety and, more particularly, for the safety of others.) If the sputum dries slowly, it may be hastened by warming the slide gently. The smear is then fixed in the usual way by passing it through the flame of the Bunsen burner several times.

Ziehl-Neelsen Method.—The Ziehl-Neelsen method of staining is the one generally employed for the tubercle bacillus.

The reagents required are:

1. *Carbol-fuchsin.*
   - *a.* Fuchsin ..... 1.0 gm.
     Absolute alcohol ..... 10.0 c.c.
     Dissolve and add—
   - *b.* 5 per cent carbolic acid..... 100.0 c.c.
2. *Acid alcohol.*
   Hydrochloric acid, conc..... 3.0 c.c.
   70 per cent alcohol to..... 100.0 c.c.
3. *Löffler's methylene blue.*
   Methylene blue, saturated alcoholic sol..... 30.0 c.c.
   0.01 per cent potassium hydrate..... 100.0 c.c.

Method.—1. Cover the specimen with carbol-fuchsin and warm it till the stain steams. Maintain this temperature for five minutes.[4] Or the

[4] The copper bar used in blood work is convenient for heating the specimen. The stain must be replenished from time to time as it evaporates, to prevent burning the specimen.

specimen may be immersed in the cold stain for twenty-four hours. It is important to overstain the preparation with carbol-fuchsin, for at best many of the bacilli are decolorized. With light staining, when only a few bacilli are present, they may be missed entirely (L. Brown).

2. Remove the excess of stain by washing in running water.

3. Decolorize in acid alcohol, until only the thickest parts of the smear have a faint pinkish tint.

4. Again wash in water. (Return to the acid alcohol if the specimen becomes pink after washing.)

5. Stain with Löffler's methylene blue 5 to 20 seconds.

6. Wash in water, dry the preparation in the air or between sheets of blotting paper. Examine in immersion oil.

The tubercle bacilli are stained red; all else is blue.

Antiformin Method for the Detection of Tubercle Bacilli.—In 1908 Uhlenhuth and Xylander[5] made the important discovery that antiformin possesses the peculiar property of dissolving all bacteria *except* those which are acid-fast, to which class the tubercle bacillus belongs. Applied to the sputum, they found that, in addition to the majority of bacteria, the great mass of the sputum is also liquefied. By the use of antiformin it is, therefore, possible to examine a large quantity of sputum and thus to concentrate the tubercle bacilli present in it. The method is valuable in those cases where the ordinary technic fails to demonstrate bacilli. A number of methods have been described for the use of antiformin, of which the following have been found serviceable:

1. Löffler's Method.[6]—The quickest method for the use of antiformin with sputum, and one which is well adapted to clinical work, has been described by Löffler. With this procedure the examination may be completed in a comparatively short time. A quantity of sputum (5 to 20 c.c.) is measured, placed in a beaker or flask of Jena glass with an equal volume of 50 per cent antiformin, and boiled. Solution of the sputum occurs almost at once, the fluid foaming and turning light brown. To 10 c.c. of the cooled solution, which is sterile, add 1.5 c.c.

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[5] Uhlenhuth and Xylander. "Antiformin, ein bakterienauflösendes Desinfektionsmittel." *Berlin. klin. Wchnschr.*, 1908, XLV, 1346.

[6] Löffler, F. "Ein neues Anreicherungsverfahren zum färberischen Nachweise spärlicher Tuberkelbazillen." *Deutsche med. Wchnschr.*, 1910, XXXVI, 1987. *Also* Williamson, C. S. "The value of the Löffler method of sputum examination," *Jour. A. M. A.*, 1912, LVIII, 1005.

of a mixture composed of 1 volume of chloroform and 9 volumes of alcohol. After shaking thoroughly, the specimen is centrifugalized for about fifteen minutes (the time varies with the speed of the centrifuge). The chloroform is thrown to the bottom of the tube, and on its surface the sediment collects. The supernatant fluid is poured off and the sediment transferred with a clean pipette to a glass slide. The excess of fluid is removed with filter paper, and a small drop of egg albumin (which may be preserved by the addition of 0.5 per cent carbolic acid) is mixed with the sediment, which is spread on the slide, fixed, and stained in the usual manner. The original sputum may be substituted for egg albumin as the fixative; it is, indeed, preferable, since a more complete examination of the sputum is possible.

The tubercle bacilli are said to be killed with this method (Löffler).

As a counterstain Löffler uses malachite green (0.1 per cent aqueous solution).

2. Paterson's Method.[7]—Paterson adds to 10 c.c. of sputum 2.5 c.c. of antiformin,[8] giving a 20 per cent strength of the latter. If the sputum is very thick or tenacious, or insufficient in quantity, a smaller amount is diluted to 10 c.c. with distilled water.[9] Solution of the sputum occurs rapidly. The mixture is poured into centrifuge tubes, which have been kept in potassium bichromate and sulphuric acid,[10] and rinsed with distilled water just before using. The tubes are stoppered with unused corks, shaken vigorously, and set aside for 24 hours at room temperature or for 4 to 6 hours at 37° C. The tubes are again

[7] Paterson, R. C. "A report on the use of 'antiformin' for the detection of tubercle bacilli in the sputum, etc." *Jour. Med. Research*, 1910, XXII, 315.

[8] The composition of antiformin, according to Paterson, is equal parts of 15 per cent solution of sodium hydrate and of liquor sodæ chlorinatæ (B. P.). The latter is prepared as follows:

Sodium carbonate	600.0 gm.
Chlorinated lime	400.0 gm.
Distilled water	4,000.0 c.c.

Dissolve the sodium carbonate in 1,000 c.c. of distilled water. Triturate thoroughly the chlorinated lime in the remainder of the water. Filter. Mix the two and filter again. There is formed an alkaline, almost colorless liquid with a strong odor of chlorin. It keeps well.

[9] Before this is done, the existence of acid-fast bacilli in the distilled water should be excluded—a troublesome source of error at times, as shown by W. Brem. *Jour. A. M. A.*, 1909, LIII, 909.

[10] Rub up some potassium bichromate with sulphuric acid for two minutes, allowing the acid to take up as much of the bichromate as it will. Pour off the acid and repeat the process.

shaken and then centrifugalized. The supernatant fluid is poured off, the tubes refilled with sterile physiological salt solution, again corked, shaken, and centrifugalized. The washing is done a second time to rid the sediment of all alkali; otherwise it does not adhere well to the glass. The sediment is then transferred to a slide, smeared, fixed, and stained by the Ziehl-Neelsen method.

The washing with salt solution may be dispensed with. The sediment, which consists of débris, swollen and distorted cells, etc., is fixed to the slide with difficulty because of the alkali, but this may be overcome by first smearing the slide with some of the original sputum or with egg white (preserved by the addition of 0.5 per cent pure carbolic acid). The original sputum is to be preferred, since the other elements present in it may thus be studied.

The *tubercle bacilli are not killed* and the sediment should, therefore, be handled with the usual precautions.

The method is valuable for obtaining material for animal inoculation or for cultures.

3. Boardman's Method.[11]—The following procedure has been found satisfactory by Boardman: Fifteen to 20 c.c. of sputum are placed in a conical specimen glass, and antiformin is added sufficient to make a 20 per cent strength of the latter. After the solution has become homogeneous and watery in consistence, an equal volume of 95 per cent alcohol is added. By this means sedimentation is facilitated, since the specific gravity of the mixture is less than 1.000. After stirring, allow it to stand till sedimentation is complete. The clear supernatant fluid is poured off (into a disinfecting solution), and smears are made of the sediment on a glass slide, using some of the original sputum as a fixative. The specimen is then fixed with heat and stained in the usual way.

Diplococcus Pneumoniae

Pneumococcus.—The pneumococcus, found not infrequently in the upper respiratory passages of healthy individuals, is conspicuous in the sputum in acute lobar pneumonia and some other acute respiratory infections.

Morphology.—According to Zinsser,[12] the morphology of the pneumococcus is one of the most useful guides to its identification. When

[11] Boardman, W. W. "The use of antiformin in the examination of the sputum for the tubercle bacillus." *Johns Hopkins Hosp. Bull.*, 1911, XXII, 269.

[12] Zinsser, H. *A Textbook of Bacteriology* (5th ed.), N. Y., 1922, p. 441.

typical, the pneumococcus is a rather large, lancet-shaped coccus, occurring in pairs, and surrounded by a definite and often wide capsule, which usually includes the two approximated cocci without a definite indentation opposite their lines of division (Fig. 54). The pneumococci may, however, occur singly or in chains, and even fairly long chains are not infrequently met with under artificial cultural conditions.

The presence or absence of capsules depends, to a large extent, upon the previous environment of the pneumococci under observation. The

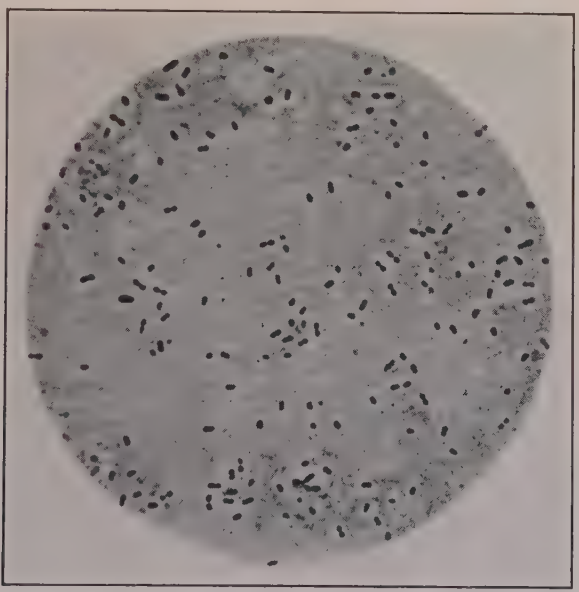

FIG. 54.—PNEUMOCOCCI STAINED TO SHOW CAPSULE. ×1000 (after Wm. B. Wherry, from photomicrograph by Chas. Goosmann).

most favorable conditions for the development or preservation of the pneumococcus capsule are found in the body fluids of man and animals suffering from pneumococcus infection. Thus, in the sputum of patients, capsules are found as a rule.

The work of R. I. Cole and his associates at the Rockefeller Hospital has shown that there are four separate types of pneumococci distinguishable by serological reactions. Types I and II are typical pneumococci morphologically and culturally. Type I organisms are agglutinated only by type I immune serum, and this serum alone protects against type I infection. Similar reactions are obtained with organisms of type II only

with type II immune serum. Type III represents what was formerly spoken of as the *Streptococcus mucosus,* but which is included in the pneumococci because of its inulin fermentation, bile solubility, and pathogenic properties which are quite similar to those of the pneumococci. Type IV is a heterogeneous group which comprises all of the true pneumococci which cannot be placed serologically in the other three groups. It is the organisms of the last group which are usually found in the throats of normal individuals.

It is of great importance to determine the type of pneumococcus in pneumonia, since the results of serum therapy in infections with organisms of type I have greatly reduced the mortality. The earlier serum therapy is begun, the greater the chance of recovery. Typing pneumococci can now be carried out with rapidity and accuracy by the method introduced by Wade Oliver.

Typing Pneumococci by Oliver's Method.[13]—The method is a rapid precipitin test on bile-treated sputum, based on the solubility of the pneumococcus in bile. After a direct smear of the sputum has been made, from 1 to 2 c.c. (up to 5 c.c., if available) [14] are placed in a clean centrifuge tube. To the sputum is then added from 3 to 5 drops of undiluted ox bile (or a 10 per cent solution of sodium taurocholate) and a sufficient quantity of sterile physiologic salt solution, if necessary, to insure a specimen of sufficient fluidity to allow of centrifugation. The mixture is then thoroughly stirred and broken up with a glass rod. It is sometimes advantageous to effect the breaking up and mixing of the sputum, bile and salt solution by grinding in a small mortar with a pestle. The tube is then heated in a water bath at a temperature of from 42 to 45°C. for 20 minutes, which time suffices for a solution of the pneumococci by the bile. The fluid is then centrifugalized. (In lieu of centrifugation, one may take a perfectly clean pipette and wrap a small amount of cotton tightly around the tip. Then immerse the tip in the sputum mixture and suck on the free end of the pipette. Usually one can draw up from 0.6 to 1.0 c.c. of clear fluid in this way).[14] Of the centrifugate (or clear fluid obtained with the pipette),[15] from 0.3

[13] Oliver, Wade W. "Further observations on a rapid method of pneumococcus typing." *Jour. Infect. Dis.*, 1921, XXIX, 518.

[14] Oliver, W. W. Personal communication.

[15] In case the fluid is obtained with the pipette, one can usually draw up from 0.6 to 1.0 c.c., which can then be distributed in three small test tubes and a precipitin test done in the manner described. Personal communication, W. W. Oliver.

to 0.5 c.c. quantities are carefully pipetted into each of three scrupulously clean tubes. To the first tube is added from 1 to 2 drops of undiluted type I pneumococcus antiserum, and, to the second and third tubes, the same quantity of type II and type III antiserum, respectively. A positive precipitin test is evidenced by an almost immediate clouding and flocculation, which is enhanced by heating at 42° C. in a water bath for from 10 to 20 minutes.

The rapid method of typing of Oliver, when it reveals a type I infection, means a saving of from 5 to 8 hours in the administration of serum, "a saving which, in certain cases, would seem to spell the difference between life and death."

Great care should be taken to obtain sputum and *not* naso-pharyngeal secretion for examination. The immune sera must be kept sterile; growth of molds in them lessens their strength.[16]

Staining.—The pneumococcus stains well with the usual anilin dyes. It is Gram-positive. The capsule may be stained with Welch's stain.

Gram's Method of Staining.

Reagents:

1. *Anilin water gentian violet.*

Ten c.c. of anilin oil are shaken with 100 c.c. of distilled water till a milky emulsion is secured. After standing five minutes, it is filtered through a *wet* filter. To the filtrate, which should contain no large oil drops, add 11 c.c. of saturated alcoholic solution of gentian violet and 10 c.c. of absolute alcohol. The solution keeps not more than 8 to 10 days (Schmorl).

2. *Gram's iodin solutions:*

Iodin	1.0 gm.
Potassium iodid	2.0 gm.
Distilled water	300.0 c.c.

Method.—1. Stain the heat-fixed smear in anilin water gentian violet 1 to 3 minutes.

2. Wash quickly in water.

3. Cover the specimen with Gram's iodin solution about 1½ minutes.

4. Decolorize in absolute alcohol until the preparation has a grayish or yellowish color—usually about 5 minutes. (The specimen may now

[16] Walker, Robert. Personal communication.

be dried and examined. If a counterstain is desired, the further steps are carried out.)

5. Wash in water.

6. Stain with 0.2 per cent aqueous solution of Bismarck brown 1 minute.

7. Wash in water, dry in the air or blot, and examine in immersion oil.

The pneumococci and all other Gram-positive organisms are stained blue, the remaining bacteria and cell nuclei are brown.

The pneumococcus or *Diplococcus pneumoniæ* grows in pairs. The long axes of the organism are placed end to end.

For demonstration of the capsules, Welch's method may be employed.

Welch's Capsule Stain.—1. Flood the fixed smear with glacial acetic acid, and immediately pour it off.

2. Wash off the acid with anilin water gentian violet.

3. Wash in 2 per cent sodium chlorid solution, and examine the wet specimen.

Bacillus Influenzae

Bacillus influenzæ (Fig. 55) is a very minute organism, which

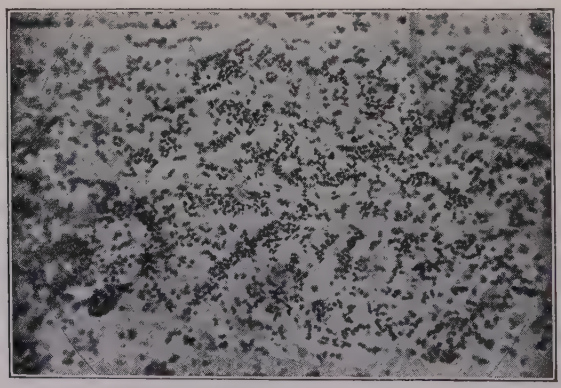

Fig. 55.—Bacillus Influenzæ. Smear from a pure culture on blood agar (after Zinsser).

measures about 0.5 micron long by 0.2 to 0.3 micron in width. At times, the bacilli exhibit a tendency to polar staining. They may be found

free in the sputum or within pus or epithelial cells. They do not form chains, and are usually found in irregular clumps in the sputum (Fig. 56), the organisms being grouped irregularly. At present, there is some doubt as to the etiological relationship of the organism to influenza.

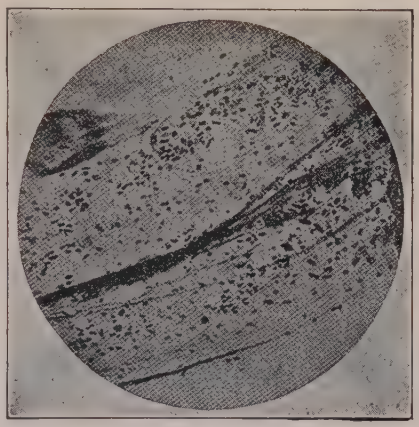

Fig. 56.—Bacillus Influenzæ. Smear from sputum (after Heim, from Zinsser).

Staining.—The organisms are decolorized by Gram's method of staining and, therefore, take the counterstain. They may be stained satisfactorily with carbolfuchsin. The stain is diluted 1:10 with distilled water, and allowed to act for ten minutes or longer. Cultural methods are required for the complete identification of *B. influenza.* It grows best on media containing blood or hemoglobin.

Bordet-Gengou Bacillus

The Bordet-Gengou bacillus, which is now generally recognized as the cause of whooping cough, is found in the sputum in almost pure culture early in the paroxysmal stage of the disease. After the first week or ten days, influenza bacilli and other secondary invaders are found in the sputum. Zinsser [17] describes the morphology and staining of the organism as follows:

Morphology.—The organism in the sputum, early in the disease, is scattered in enormous numbers indiscriminately among the pus cells, and at times within the cells. It is extremely small and ovoid, and frequently is so short that it resembles a micrococcus. Often its poles stain more deeply than the center. In general, the form of the organisms is constant, though occasionally slightly larger individuals are encountered. They are usually grouped separately, though occasionally in pairs, end to end. Compared with the influenza bacillus in morphology, the bacillus of pertussis is more regularly ovoid and somewhat

[17] Zinsser, H. *A Textbook of Bacteriology,* N. Y., 1922 (5th ed.), p. 505.

larger. It has, furthermore, less tendency to pleomorphism and involution.

Staining.—The Bordet-Gengou bacillus may be stained with Loeffler's methylene blue, dilute carbol-fuchsin, or aqueous fuchsin solutions. It is Gram-negative. Bordet and Gengou recommended as a staining solution carbolated toluidin blue made up as follows:

Toluidin blue	5.0 gm.
Alcohol	100.0 c.c.
Water	500.0 c.c.

Dissolve, and then add 500.0 c.c. of 5 per cent carbolic acid. Allow the mixture to stand one to two days and filter.

Actinomyces Bovis

Actinomyces bovis (Fig. 57) (Syn.: *Nocardia bovis, Actinomyces hominis, Streptothrix hominis*), the ray fungus, which is the causative agent in "lumpy jaw" of cattle, is occasionally parasitic in man. The lungs are often the site of infection.[18] The sputum is characteristic. Claypole studied the series of cases reported by Bridge, and describes the sputum as follows: "In the majority of cases the sputum is characteristic and of two types: (1) glairy, mucilaginous, often quite watery; (2) purulent, more or less bloody, more or less—sometimes intensely—fetid. Both types may be found sparingly or in abundance. . . . The small granules (of the fungus), usually the size of a very small pinhead, can be picked out with a needle and put on a slide for examination. They are quite tough, and can be washed free of débris by putting them in a dish of water and squirting them vigorously up and down a pipette. . . .

Fig. 57.—Actinomyces Hominis. Showing club-shaped extremities to the rays. Fresh preparation (after Wood).

"Under low magnification the yellow color is marked; to the naked

[18] For a review of the subject, see Bridge, N. "Streptothricosis (actinomycosis) of the lungs." *Jour. A. M. A.*, 1911, LVII, 1501.

eye the fungus is grayish-white. The edge is always darker, even shading into a brown; toward the center it grows lighter. From this light, almost homogeneous center, the characteristic radiations arise. Higher magnification shows the center to be a mass of pale, radiating threads, the mycelia, and at the edges a mass of threads and cocci. Both mycelia and cocci may be stained with methylene blue, the former frequently being banded light and dark in segments, sometimes granular throughcut."

Streptothrix Eppingeri

Streptothrix eppingeri (Syn.: *Nocardia asteroides*) is an organism related to the preceding. In the case described by Warthin and Olney [19] a filamentous, branching organism was found. Usually the mycelia were tangled and interwoven. No conical or club-shaped terminations were found, as in *Actinomyces bovis*. The threads, stained with carbolfuchsin, were not decolorized after treatment with 25 per cent nitric or sulphuric acids, though the stain was largely removed by washing in 95 per cent alcohol.

Blastomycetes

Blastomycetes (Fig. 58) have been found in the sputum in a number of cases. "In unstained preparations of pus and tissue, the organisms appear as round or oval bodies with a double-contoured, highly refractive capsule. Within the capsule, in many instances, granules or sporelike bodies can be distinguished. The addition of a 1 to 10 per cent solution of potassium hydrate to the specimen under examination, facilitates the recognition of these bodies. In stained sections the double-contoured, homogeneous capsule is usually separated from a finely or coarsely granular protoplasm by a clear space of varying width. Vacuoles of different sizes are found in some organisms. In both pus and tissue, organisms in pairs or in various stages of budding are commonly seen. The parasite, as a rule, varies in size from 7 to 20 micra, though slightly smaller and much larger forms occur in some cases." [20]

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[19] Warthin, A. S. and Olney, H. S. "Pulmonary streptothricosis." *Amer. Jour. Med. Sci.*, 1904, CXXVIII, 637.

[20] Montgomery, F. H. and Ormsby, O. S. "Systemic blastomycosis." *Arch. Int. Med.*, 1908, II, 1. See also Hektoen, L. "Systemic blastomycosis and coccidioidal granuloma." *Jour. A. M. A.*, 1907, XLIX, 1071. (Literature.)

Spirochetes

Treponema bronchiale (Syn.: *Spirochaeta bronchialis*, *Spiroschaudinnia bronchialis*).[21]—The organism responsible for spirochetal bronchitis,

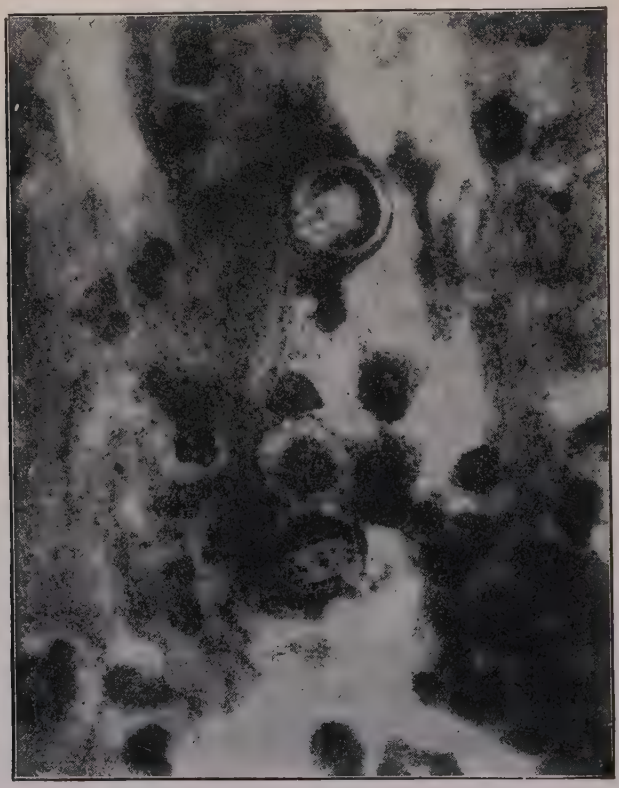

Fig. 58.—Blastomycetes in Sputum. ×1500. Photomicrograph (after E. E. Irons and E. A. Graham).

first described by Castellani in 1905 and 1906, may be found in the sputa in large numbers. The spirochete is very polymorphic, varying in length, thickness, and the number of waves in the spiral. The length

[21] Castellani, A. and Chalmers, A. J. *Manual of Tropical Medicine*, (3d ed.), N. Y., 1919, p. 1883; *also* Bloedorn, W. A. and Houghton, J. E. "Bronchial spirochaetosis." *Jour. A. M. A.*, 1921, LXXVI, 1559.

may vary between 5 and 30 micra, the breadth between 0.2 and 0.6 micron. The ends vary in shape, but are generally acuminate. The number of spirals varies between two and eight. In fresh preparations, *S. bronchialis* is actively motile for only a short time, thus differing, as shown by Chalmers and O'Farrell, from the oral spirochetes, which live for hours outside the mouth. Fantham has shown that the motile phase is succeeded by one of granule formation, the granules or coccoid bodies representing a resting stage, from which new spirochetes develop.

The sputum for examination should be fresh; the teeth, mouth and throat should be cleansed with an antiseptic solution to exclude, so far as may be, spirochetes from these sources.

The spirochetes may be demonstrated in the fresh specimen by means of the dark field illumination; or smears of the sputum may be made and stained by Fontana's method or by Giemsa's, Leishman's, Wright's or Wilson's stain.

ANIMAL PARASITES IN THE SPUTUM

In this country, animal parasites are comparatively rare in the respiratory passages, though probably more common than is generally supposed.

Entameba dysenteriae (Fig. 24, p. 166), may be encountered in the sputum as the result of rupture of an amebic liver abscess into the bronchial tree. The organism is identical with that found in the feces (q. v.). Perfectly fresh sputum should be examined—when possible, with a warm stage.

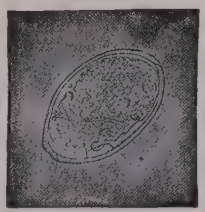

FIG. 59.—OVUM OF PARAGONIMUS RINGERI. From the sputum. ×400 (after Emerson).

Trichomonads (Fig. 25, p. 168) are occasionally found in the sputa, usually in material which has stagnated in the lung.

Paragonimus ringeri, the lung-fluke, the cause of "parasitic hemoptysis," is rare in this country, common in Japan and parts of China. Diagnosis is made by finding the ova (Fig. 59) in the fresh sputum (see p. 204).

Echinococcus cyst, though common in certain parts of the world, is excessively rare in this country. Diagnosis may be made after rupture of the cyst into the bronchi by (1) the finding of daughter cysts, (2) scolices, (3) hooklets, or (4) parts of the membrane in the sputum

(Fig. 60). The material may arise from a hepatic cyst which has ruptured through the diaphragm into the air passages.

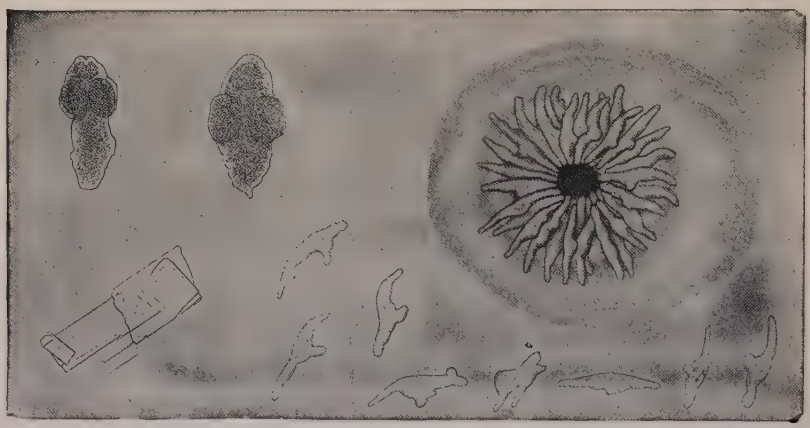

FIG. 60.—SEDIMENT FROM ECHINOCOCCUS CYST. Above and to the left are two degenerated scolices (× about 60); to the right is a crown of hooklets (×400); below are hooklets of unusual shapes and a small mass of cholesterin crystals (×400) (after Emerson).

CHAPTER V

SECRETIONS OF THE MOUTH, THROAT AND NOSE

The study of the secretions of the mouth, throat and nose is limited almost entirely to the more important microörganisms encountered in health and disease. It is often quite as important to detect pathogenic organisms in normal individuals (carriers) as to determine the etiology of a disease process. The more detailed and complicated methods for the identification of pathogenic microörganisms are not discussed in the following pages.[1] Only the methods of examination which any well-trained practicing physician can himself apply to advantage are considered.

DIPHTHERIA

Cultures may be desired for two reasons, (1) the detection of carriers and (2) the determination of the etiology of an acute inflammatory process in throat or nose.

For the detection of *carriers,* secretion from the tonsillar and posterior naso-pharyngeal regions is obtained on sterile swabs. Tubes of blood serum are inoculated and incubated (see below), and direct smears on glass slides may then be prepared for microscopic examination. The examination of the direct smears is untrustworthy for diagnosis, and reliance should be placed rather on the results of cultures.

In any case of *acute inflammation* of the nose and throat, cultures should be made as early as possible, and if positive for *B. diphtheriae,* antitoxin should be administered at once, without waiting for the appearance of the membrane.

Obtaining Material for Cultures.—It is important that the sterile swab, used for obtaining the material for culture, should not come in contact with other mucous membrane than the one to be examined. To

[1] Zinsser, H. *A Textbook of Bacteriology* (5th ed.), N. Y., 1922, and Park, W. H. and A. W. Williams' *Pathogenic Microörganisms* (7th ed.), Phila. and N. Y., 1920, are good reference books.

avoid such contact, it is advisable to use a tongue depressor, and the throat should be well illuminated. In making smears from the nasopharynx, a swab mounted on a wire which may be bent to form a suitable angle is a convenience. The swab is drawn over the inflamed surface —if a false membrane is present, material should be obtained from it— and tubes of *Loeffler's blood serum* [2] are inoculated and *incubated for 9 to 18 hours* (*not longer*) at 37° C. (98.6° F.). If there is likely to be delay in placing the tubes in the incubator, they may be carried in the inside vest pocket until transfer to the incubator can be made.

From a fresh culture, of the age stated above, *smears* are made with a platinum loop upon glass slides, allowed to dry in the air, and *fixed* with heat by passing the slide through the flame of a Bunsen burner three times. Smears should also be made directly from the swab *after* the culture media have been inoculated. (It is better to employ a separate swab for making direct smears.) The specimens are then stained, using Albert's method.

Since the appearance of diphtheria bacilli from cultures is a matter of great importance in laboratory diagnosis, the detailed description of Graham-Smith [3] is quoted in great part.

In stained preparations made from colonies 9–18 hours old growing on Loeffler's serum medium, diphtheria bacilli vary greatly in appearance and may belong to several types. For the sake of convenience and clearness, the appearances are described under various headings, as follows:

Arrangement.—The bacilli may occur singly, but are more usually arranged in groups of three, four or more individuals lying side by side at a more or less open angle with each other, or occasionally one individual may lie across the others. Sometimes the bacilli lie end to end, but always at a more or less open angle with each other, resembling the letters L or V. One small group of bacilli, the members of which are lying more or less parallel, is often found near a similar small group, forming a mass with interlacing ends. The arrangement of the bacilli in the field has also been compared to pine-needles lying on the ground. Chains even of moderate length are never found (Fig. 61).

Size.—Diphtheria bacilli from serum cultures vary greatly in length, and have been divided according to their size into long, short, and

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[2] Sterile swabs and Loeffler's serum in tubes are supplied gratis to physicians by State and Municipal Health Departments.

[3] Graham-Smith, G. S. *The Bacteriology of Diphtheria* (edited by G. H. F. Nuttall and G. S. Graham-Smith). Cambridge, 1908, p. 125.

medium length bacilli. The diameter of the bacilli varies between 0.2 and 0.8 micron, and the length usually between 1 and 8 micra, but, exceptionally, even longer forms up to 13 micra occur.

Shape.—The individual bacilli are usually slightly curved, but greatly curved forms are frequently encountered. Straight bacilli are seldom met with.

The bacilli are seldom, if ever, uniformly cylindrical throughout their entire length, but are usually somewhat swollen at one end or the other, and not infrequently at both, especially in the longer forms. Slighter irregularities are generally present throughout their length. The ends

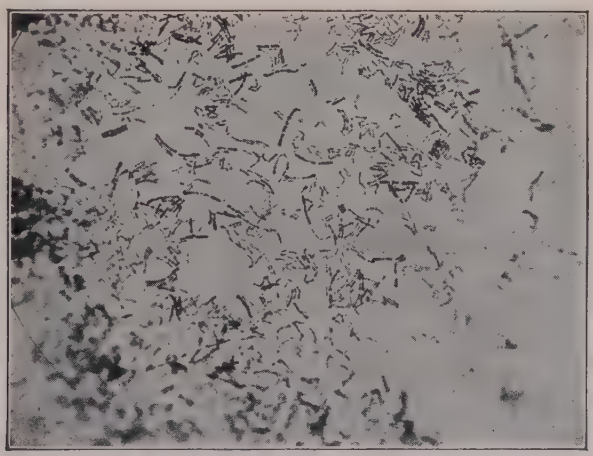

FIG. 61.—BACILLUS DIPHTHERIAE (after Zinsser).

are usually rounded, but specimens are occasionally met with in which one end is more or less pointed.

Diphtheria bacilli may be *classified* according to their shape and size. Amongst the *shorter* forms wedge-shaped and irregular specimens are the most common. In these forms the swelling usually occupies one-half of the bacillus, and is not entirely confined to the end, the other half being distinctly smaller and sometimes having a pointed extremity. Irregular forms with the swelling near the center, or without any marked swelling, are met with among these forms. In the *longer* forms wedge-shaped specimens are not common. The organism is gently curved and the ends are usually slightly greater in diameter than the

rest of the bacillus, and one or more slight irregular swellings may be present in its length. These bacilli usually have a length of 3-4 micra. A third group consists of bacilli of about the same length or decidedly longer which have either one end or both decidedly swollen. The swollen ends are frequently twice as broad as other parts of the bacilli. Such bacilli are spoken of as *club-shaped.*

Short oval bacilli, which are young forms, may be encountered in almost any culture.

In preparations from any given culture the majority of the bacilli are usually found to belong to one of these three groups, the last two of which are of more common occurrence than the first.

Staining of the Protoplasm.—Diphtheria bacilli have also been classified according to the mode in which their protoplasm stains by the common dyes, especially methylene blue. When stained by Loeffler's methylene blue solution, some portions of the protoplasm usually stain deeply and others lightly. *The most typical condition is that which is usually met with in the long and club-shaped forms. In these, the terminal swellings are usually deeply stained and frequently show in them violet-tinted granules.* The rest of the protoplasm in this type is unevenly stained, showing alternate, irregular, lightly and darkly stained bands. These bands are seldom transversely placed across the length of the bacillus, but cross the long axis of the organism at various angles. Such specimens may be described as *banded* or *segmented* bacilli. *Another type of diphtheria bacillus is faintly stained but has irregular patches of darkly stained protoplasm in its substance. These have been termed irregular beaded bacilli.* In a third type the darkly stained parts are rounded and disposed regularly along the length of the bacillus, giving it the appearance of a short chain of streptococci. *Of the above three types, the first two are by far the most common, and are the ones described by most observers as typical of the diphtheria bacillus in culture.* In a fourth type, which is less frequently met with, the bacilli are uniformly and darkly stained throughout their length. Lastly, *oval* bacilli uniformly stained or with a light central band (very young forms) may sometimes be seen among the other forms.

The darkly stained violet granules (polar bodies) mentioned above may be met with in all of these types except the very young forms.

Interpretation of the Result of Culture.—The finding of organisms, which have the morphological and staining characteristics described above in cultures incubated not more than 18 hours or in direct smears from

the throat and nose, means, in the great majority of instances, that *B. diphtheriae* is present. Such a finding does *not* necessarily mean that the patient has diphtheria. The organisms may be virulent or avirulent diphtheria bacilli from a carrier, or diphtherialike bacilli, such as *B. hofmanni.*

Virulence Test.[4]—It is important to determine the virulence of the bacilli when diphtherialike organisms have been found in a healthy individual or when they persist beyond the usual convalescent period in one recovered from the disease. This is most conveniently performed by washing off the growth from a culture with about 10 c.c. of sterile salt solution and injecting 1 c.c. of this suspension into a guinea pig *subcutaneously.* If virulent bacilli are injected in this way along with the mixed culture from the throat or nose, the guinea pig will die in from 2 to 4 days; and upon dissection the serogelatinous subcutaneous oedema and markedly congested adrenals characteristic of such infection in guinea pigs will be found. To make the diagnosis still more certain, two guinea pigs, one of which is given 100 units of antitoxin, may be used.

If an *acute inflammation* of the throat exists, the case should be treated as one of diphtheria, and antitoxin should be administered without delay, since the results are far better when it is given early. With a *false membrane,* a positive culture means diphtheria in the majority of instances, but not in all; patients with a streptococcus sore throat, for example, may be, at the same time, diphtheria bacillus carriers.

The best of the staining methods is Albert's.

Albert's Staining Method.[5]

Reagents:

1. Toluidin blue	0.15	gm.
Methyl green	0.20	gm.
Acetic acid (glacial)	1.0	c.c.
Alcohol (95 per cent)	2.0	c.c.
Distilled water	100.0	c.c.

After standing for one day, the solution is filtered and is ready for use.

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[4] Wherry, Wm. B. Personal communication.

[5] Albert, H. "Modification of stain for diphtheria bacilli." *Jour. A. M. A.,* 1921, LXXVI, 240.

2. Iodin ..... 2.0 gm.
   Potassium iodid ..... 3.0 gm.
   Distilled water ..... 300.0 c.c.
   The solution is ready for use as soon as the iodine is entirely dissolved.

Method.—Smears are made on slides or cover glasses and fixed by heat in the usual manner.

1. Stain with solution (1) for one minute.
2. Dry with good absorbent filter paper.
3. Stain with solution (2) for one minute.
4. Wash, and dry with filter paper.
5. Examine with oil immersion objective.

The granules of diphtheria bacilli are stained black; the bars dark green, and the intermediate portions a light green. Virtually all other bacteria also take a light green stain. The contrast is marked.[6]

Neisser's Staining Method.

Reagents:

1. Methylene blue ..... 1.0 gm.
   Alcohol, 90 per cent ..... 20.0 c.c.
   Dissolve and then add—
   Distilled water ..... 950.0 c.c.
   Acetic acid, glacial ..... 30.0 c.c.

2. Vesuvin (Bismarck brown) ..... 2.0 gm.
   Boiling distilled water ..... 1,000.0 c.c.
   Dissolve. Filter after the solution cools.

Method.—1. Stain in methylene blue solution 1 to 3 seconds.
2. Wash quickly in water.
3. Stain in vesuvin 3 to 5 seconds.
4. Wash quickly in water, blot dry, and examine in oil.

[6] Dr. G. M. Guest, who has made a comparative study of a large number of stains for *B. diphtheriae* in the laboratory of the Contagious Department of the Cincinnati General Hospital, has found Albert's stain to be the most satisfactory. With it, the results obtained by *examination of the direct smear* have been confirmed by culture in about 80 per cent of cases. (Personal communication).

The diphtheria bacilli are slender rods, often having a slight bend. They are stained light brown, with one to three granules, which take a dark blue color. Parallel pairs of bacilli are characteristic. Chain formation is lacking. Other bacteria, which are present in the smear, are stained light brown, so that the blue polar bodies of *Bacillus diphtheriae* are striking and characteristic.

Beall's Method.[7]—The polar bodies may be demonstrated well by Beall's method.

1. Overstain the specimen with anilin water gentian violet ¼ to ½ minute.
2. Wash in water.
3. Decolorize with 10 per cent glacial acetic acid till little color remains. This is controlled under the microscope.
4. Wash in water, blot dry, and examine in immersion oil.

All Gram-negative organisms are decolorized, and most of those which are Gram-positive. The polar staining is intense, and diphtheria bacilli stand out prominently. Practically all other organisms are decolorized more or less completely.

FALSE MEMBRANES ASSOCIATED WITH OTHER ORGANISMS

That other organisms than *B. diphtheriae* may be associated with false membranes in the mouth, nose and larynx is a well known clinical fact. The local lesion may be indistinguishable clinically from that of diphtheria. The organisms most frequently found associated with non-diphtheritic membranous lesions of the mouth, nose and larynx, according to Graham-Smith,[8] are the following:

Streptococci.—In many cases with false membrane, streptococci (Fig. 62) may be found in practically pure culture. Woodhead [9] found streptococci to be the only organisms present in 565 out of 1,960 cases diagnosed on clinical grounds as diphtheria, in which no evidence of diphtheritic infection was found.

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[7] An unpublished method of Dr. H. K. Beall of Fort Worth, Texas, through whose kindness it is given here.

[8] Graham-Smith, G. S. *The Bacteriology of Diphtheria* (edited by G. H. F. Nuttall and G. S. Graham-Smith). Cambridge, 1908, p. 391.

[9] Woodhead, G. S. "Report of the bacteriological diagnosis and the antitoxic serum treatment of cases admitted to the hospitals of the Board during the years 1895 and 1896." *Metropol. Asylums Board,* London, 1901. (Cited by Graham-Smith.)

Staphylococci.—Instances of membranous inflammation due to staphylococci are by no means uncommon.

Mixed Infection *with streptococci and staphylococci* is a commoner cause of pseudo-diphtheritic inflammation than pure infection with either of the organisms, according to Woodhead, who found 985 of his 1,960 cases of pseudo-diphtheria due to mixed infection with these two organisms.

Pneumococcus.—Membranous inflammation of the fauces may occur

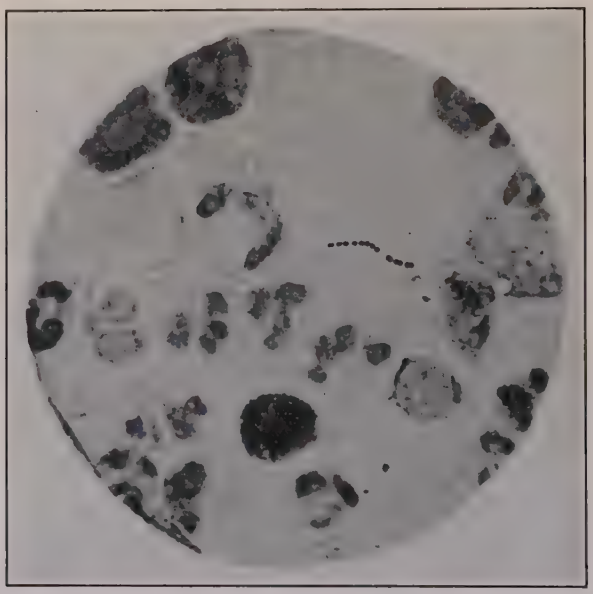

Fig. 62.—Streptococci in an Exudate. ×1000 (after Wm. B. Wherry; photomicrograph by Chas. Goosmann).

during the course of a pneumonia or unassociated with pneumonia, in which pneumococci may be found in pure culture (Fig. 54). The membrane is very extensive at times, involving the tonsils, pillars of the fauces and uvula; the pharynx and nasal mucous membranes may also be attacked.

Vincent's Angina.—Smears may be taken directly from the lesions, and after heat fixation they are stained with gentian violet (see Gram's stain, p. 217). The specimen is examined for the *fusiform bacilli* and the *Spirochaeta vincenti.* The false membrane may be situated in the

pharynx, on the tonsils or the mucous membranes of the mouth. The *fusiform bacilli* (Fig. 63) are from 3 to 10 micra long and have a thickness at the center varying from 0.5 to 0.8 micron, according to Zinsser,[10] and they taper gradually toward the ends, ending in blunt or sharp points. The length may vary greatly in a given smear. They are usually straight, sometimes slightly curved. They are best stained with Giemsa's stain. The bacilli are more deeply stained near the ends and have a banded or striped alternation of stained and unstained areas in the central part of the bacilli, resembling somewhat in this respect diphtheria bacilli. The bacilli are nonmotile. *Spirochaeta vincenti* (Fig. 63) is

FIG. 63.—THROAT SMEAR. Vincent's angina. Fusiform bacilli and spirochætes (after Zinsser).

very actively motile and measures 10 to 20 micra in length, though shorter or longer individuals are met with. The spirals are few in number and are elongated.[11] The organisms are well demonstrated with dark field illumination, and with Giemsa's or Fontana's stains.

Other organisms have been found in a few instances in false membranes, either in pure culture or in mixed infections. Thus, the *Friedländer bacillus*, *Bacillus coli*, and the *Brisou coccus* have been encountered occasionally. In the tropics, *Monilia tropicalis* is at times the cause of a pseudo-diphtheria.

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[10] Zinsser, H. *A Textbook of Bacteriology* (5th ed.), 1922, p. 868.

[11] Castellani, A. and Chalmers, A. J. *Manual of Tropical Medicine* (3d ed.), 1919, p. 448.

Diphtheria-like Bacilli

In the secretions of the mouth and nose in health and disease, organisms resembling diphtheria bacilli, which are non-pathogenic, may be found. Of these, the most important is *Bacillus hofmanni.* The organism is shorter and thicker than the diphtheria bacilli, and may be differentiated culturally by the fact that the Hofmann bacillus cannot form acid from sugars added to culture media, whereas *B. diphtheriae* acidifies and coagulates media containing monosaccharids. The virulence test will also serve to differentiate.

PROTOZOA

Entameba gingivalis (Syn.: *E. buccalis; E. dentalis; E. maxillaris*) is found in the pus from pyorrhea pockets and has been looked upon as the cause of pyorrhea alveolaris, though it is not yet proved that the organism is pathogenic. Owing to the great frequency with which it is found in the mouth, the ameba may at times be encountered in the sputum through admixture. In disease, *E. gingivalis* has been encountered only in the oral cavity, usually, as already stated, in connection with pyorrhea alveolaris.

The vegetative forms of the parasite are 7 to 35 micra in diameter, the average being 12 to 20 micra. Ameboid motion is quite active, and it is only when actively motile that the ectoplasm of the ameba is well defined; short, blunt or long, tapering pseudopodia are thrown out. The endoplasm is granular and contains non-contractile food vacuoles and often red blood corpuscles. Generally, the nucleus is not visible in the unstained specimen. *Cysts* are formed; they contain one nucleus and measure 8 to 10 or more micra in diameter.[12]

The amebae are *detected by examination of the fresh, unstained pus* expressed from a pyorrhea pocket. A small drop or a loopful of the pus is transferred to a glass slide and a cover glass is placed on it. The specimen should be examined at once.

Leishmania tropica (see p. 346) is found in oral leishmaniasis and in espundia or naso-oral leishmaniasis of South America.

Oidium albicans (Syn.: *Monilia, Saccharomyces, Endomyces*) (Fig. 64) is generally stated to be the cause of *thrush,* though Castellani has shown that a number of fungi may cause the disease. The organism forms

[12] Castellani, A., and Chalmers, A. J. *Manual of Tropical Medicine* (3d ed.), N. Y., 1919, p. 317.

white patches on the tongue and oral mucous membranes, which are easily detached. According to Castellani and Chalmers, *Oidium albicans* in a particle of the patch has the following characters: There are septate mycelial threads, occasionally ramified, with segments straight or somewhat bent, and easily dissociated. Each segment is about 20 micra in length and 3 to 5 micra in breadth. At the terminal end of each mycelial thread, three or four shorter ovoid elements are found, which reproduce by budding. Some similar ovoid or roundish, globular, refringent cells

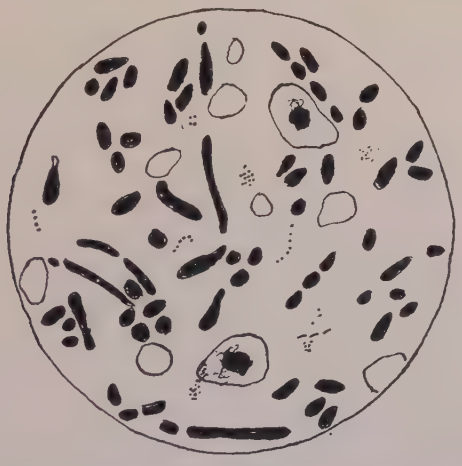

FIG. 64.—OIDIUM ALBICANS FROM A CASE OF THRUSH (after Deguy and Guillaumin, from Laroche).

may be observed originating laterally at the septations of the mycelium. These globular elements (conidia) become detached, and reproduce by budding.

Blastomycosis (p. 221) may involve the oral mucous membranes. The organisms are similar to those found in the sputum.

SPIROCHETES

Spirochetes are normal inhabitants of the mouth. They are commonly found in the pus of pyorrhea alveolaris, about the tartar of the teeth, etc. *Treponema mucosum, T. microdentium* and *T. macrodentium* have been described by Noguchi. The organisms are well demonstrated by dark field illumination and may also be detected in stained smears. *Spirochaeta dentium* and *S. buccalis,* also non-pathogenic, are normally present.

CHAPTER VI

CONJUNCTIVAL EXUDATES

Inflammations of the conjunctiva are caused by various microörganisms. There is a mucopurulent or purulent secretion, in which the organisms are usually demonstrable, either by direct staining of the smears, or by cultures, or by a combination of the two.

Gonococcus.—In ophthalmia neonatorum, the gonococcus (Fig. 17, p. 103) is the usual organism. Conjunctivitis in older children and in adults is also not infrequently caused by the same organism. (For the details of demonstrating the gonococcus, see p. 102 *et seq.*)

Streptococcus.—In the course of an eruptive fever, a conjunctivitis caused by streptococci may occur.

B. Diphtheriae.—The etiological diagnosis is made in the manner described on page 225 *et seq.*

The *exudate* in the foregoing instances is generally abundant and is *purulent.*

Koch-Weeks Bacillus.—This organism (Fig. 65) causes an acute conjunctivitis which occurs in epidemics ("pink eye"). The bacillus resembles the influenza bacillus morphologically, though generally longer and more slender (Zinsser). It is Gram-negative. The bacilli may be found within the leukocytes as well as extracellularly. The exudate is mucopurulent.

Morax-Axenfeld Bacillus.—This organism was originally discovered in France by Morax, whose findings were later confirmed by Axenfeld. It is met with in certain cases of chronic conjunctivitis, with mucopurulent exudate. In smear preparations from the pus, the microörganisms appear as short, thick diplobacilli, though not infrequently they are found singly or in short chains. Their ends are distinctly rounded, their centers slightly bulging, giving the bacilli an ovoid form. They are usually about two micra long and are Gram-negative (Zinsser).

Pneumococci, staphylococci, meningococci, and *Micrococcus catarrhalis* are occasional causes of conjunctivitis.

Micrococcus catarrhalis is a diplococcus which is indistinguishable morphologically from the meningococcus and the gonococcus. It is Gram-negative, and when found, is often intracellular. It is readily differentiated from the gonococcus by the fact that it grows on simple culture

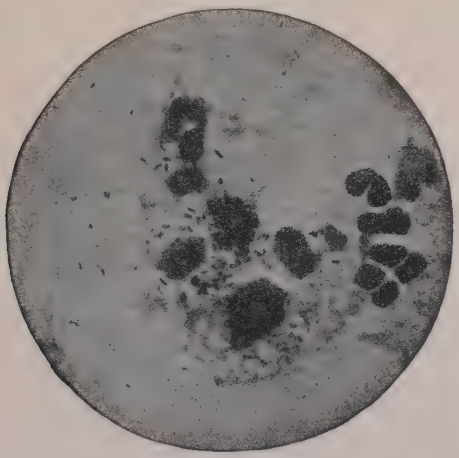

Fig. 65.—The Koch-Weeks Bacillus (after Zinsser).

media. Differentiation from the meningococcus is not so easy; it is based entirely upon cultural differences and agglutination reactions.

B. tularense is a rare cause of ulcerative conjunctivitis.[1]

OPHTHALMOMYCOSES

Diseases of the eye and its adnexa may be caused by various fungi.[2]

Blastomycosis.—This affects the lids. The causal agent is *Cryptococcus dermatitis.* Diagnosis is made by culture of the fungus.

Tineae.—Tinea palpebrarum may be caused by *Microsporon lanosum* and by various species of *Trichiphyton,* of which that most commonly found is *T. tonsurans.* The diagnosis is made by culture of the fungus. *Achorion schoenleini,* the fungus of favus, may also attack the eyelids.

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[1] Wherry, Wm. B., and Lamb, B. H., "Infection of man with Bacterium tularense," *Jour. Infect. Dis.*, 1914, XIV, 331.

[2] From Castellani, A., and Chalmers, A. J. *Manual of Trop. Med.* (3d ed.), 1919, p. 2009.

Aspergillosis.—Infection with *Aspergillus fumigatus* may be diagnosed by microscopical or cultural examination of scrapings from the ulcer or of the pus from an abscess.

Nocardiases.—Actinomycotic conjunctivitis is met with occasionally. The organisms which have been encountered are *Nocardia bovis, N. israeli, Cohnistreptothrix foersteri* and *N. dassonvillei.*

Moniliasis.—*Monilia albicans,* one of the organisms of thrush, may very rarely attack the conjunctiva. In the tropics, cases due to *M. tropicalis* have also been observed.

Glenosporosis.—One case, due to *Glenospora graphii,* has been described by Morax.

Sporotrichosis.—Sporotrichal infection of the eyelids, conjunctiva, lachrymal sac and iris have been recorded. The causative agent has been *Sporotrichum beurmanni.*

ANIMAL PARASITES

Paragonimiasis.—Cysts containing *Paragonimus ringeri* have been reported. They have been found on the eyelids and in the orbit.

Filariasis.—*Loa loa*[3] (Syn.: *Filaria oculi; Dracunculus oculi; F. subconjunctivalis*) has been found in the ocular and palpebral conjunctiva. It also occurs in the subcutaneous fat and the superficial aponeuroses in all parts of the body. Infection is diagnosed by finding the adult parasites in the conjunctiva or by the detection of the larvae, *Microfilaria diurna,* in the circulating blood (see p. 351).

Other parasites which have been encountered, according to Castellani and Chalmers, are *Agamodistomum ophthalmobium* and *Monostomum lentis,* both of which may be stages of *Dicrocoelium lanceatum; Agamofilaria oculi humani; Agamofilaria palpebralis; Dermanussus gallinae,* which may be seen as a dark spot embedded in the cornea; *Demodex folliculorum,* which is found in the Meibomian glands, and may cause a blepharitis.

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[3] Begle, H. L. "Infestation with Filaria loa. Report of a case of filaria beneath the conjunctiva and microfilariae in the peripheral blood stream." *Jour. A. M. A.,* 1921, LXXVI, 1301.

CHAPTER VII

VEGETABLE AND ANIMAL PARASITES OF THE SKIN

The microörganisms concerned in the production of various skin lesions, such as the streptococci, staphylococci, etc., differ in no essential particulars from those already described in connection with disease processes elsewhere in the body. There are, in addition, certain fungi and animal parasites, which may be found in or on the skin, which are of diagnostic importance.

ANIMAL PARASITES

Treponema pallidum, the cause of syphilis, has been described on page 104.

Leishmania tropica is the causative agent in cutaneous leishmaniasis (see page 346).

Sarcoptes scabei (Syn.: *Acarus scabei*) is the causative agent in scabies. The female burrows beneath the epidermis, and the diagnosis is made by finding the mite. The female deposits feces and ova (about 0.15 mm.) along the burrow, while the mite itself lies buried in the horny layer at the far end of the burrow and just beyond a small vesicle, which often marks its position. It may be obtained by opening the burrow with a fine needle. The mite is extracted, transferred to a glass slide, covered with a small drop of water and examined microscopically. Its length varies from 0.33 to 0.45 mm. and its breadth from 0.25 to 0.35 mm. (Fig. 66).

Pediculus capitis, the head louse, lives on the hairs of the head, at times on the eyebrows or beard, and exceptionally on the pubic hair. The size varies, the average being for the males 1.6 by 0.7 mm. and for the females 2.7 by 1 mm. (Fig. 67). The females deposit their ova or nits on the hairs; the ova (Fig. 68) are about 0.6 mm. in their long diameter. Diagnosis is made by finding the parasites or their ova. They are visible macroscopically.

Pediculus vestimenti (Syn.: *P. corporis*), the body louse, is larger than the head louse. The males average 3 by 1 mm., the females 3.3

by 1.2 mm. (Fig. 69). They are found on the body only when feeding, at other times being on the undergarments. The ova are 0.7 to 0.9

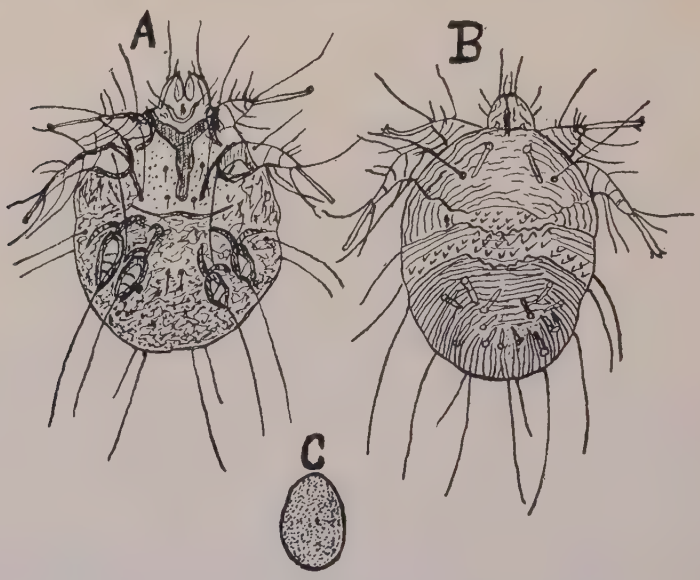

Fig. 66.—Sarcoptes Scabiei var. Hominis. Female. *A*, ventral surface; *B*, dorsal surface; *C*, ovum. ×100 (after Neveu-Lemaire).

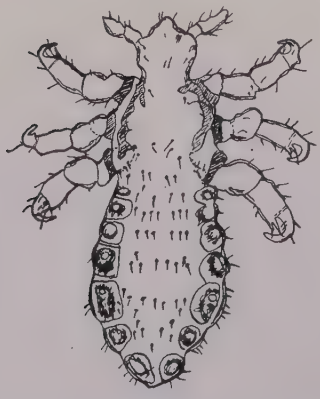

Fig. 67.

Fig. 68.

Fig. 67.—Pediculus Capitis. ×28 (after Braun).
Fig. 68.—Ovum or Nit of Pediculus Capitis on a Hair. ×70 (after Braun).

mm. long and, like the adult parasites, are found on the underclothes on the side of the garment which is next to the skin. Aside from the

skin lesions produced, the pediculi are important as *vectors* in the spread of disease. Thus, *relapsing fever, typhus fever* and *trench fever* are known to be transmitted by the body louse. The parasites are grayish in color.

Pediculus pubis (Syn.: *P. inguinalis; Phthirius inguinalis*), the crab louse, lives in the suprapubic hair. The average dimensions of the males are 1.3 by 0.8 mm., of the females 1.5 by 1 mm. (Fig. 70). They are gray or grayish-yellow in color. The eggs are 0.8 to 0.9 mm. by about

Fig. 69.

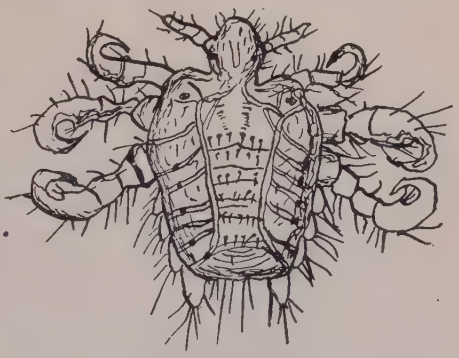

Fig. 70.

Fig. 71.

Fig. 69.—Pediculus Vestimenti. ×28 (after Braun).
Fig. 70.—Pediculus Pubis (after Braun).
Fig. 71.—Pulex Penetrans. Free female. ×20 (after Karsten from Neveu-Lemaire).

0.5 mm. and are found on the hairs. Occasionally, the parasites are found on other hairy parts of the body, such as the abdomen, the chest, the axillae, the beard or the eyebrows; on the head, they are excessively rare. This parasite is also suspected of being a vector of typhus and relapsing fevers.

Sarcopsylla penetrans (Syn.: *Pulex penetrans; Rhynchoprion penetrans; Dermatophilus penetrans*) is the sand-flea or jigger. The females penetrate the skin and are found on the hands, wrists, knees and especially on the feet. The parasite is about 1 mm. in diameter (Fig. 71).

Dracunculus medinensis (Syn.: *D. veterum; D. persarum; Vena medinensis; Filaria dracunculus; F. aethiopica*), the guinea worm, is endemic in tropical Africa, India, Persia, Turkestan, Arabia and in some parts of South America, to which it was imported from Africa. The female, after becoming gravid, leaves the connective tissue about the mesentery and moves downward as a rule to the leg or foot, occasionally to the arm or hand, rarely to the head. A small bulla usually marks the presence of the worm beneath the skin, or the outline of the worm may be visible under the epidermis. The female is a long white filiform worm 50 to 80 cm. long and 0.5 to 1.7 mm. in thickness. The parasite is delicate and considerable care is required in extracting it.

Onchocerca volvulus (Syn.: *Filaria volvulus*) is endemic in tropical Africa in the Gold Coast and particularly in the Congo. The adult parasites form subcutaneous nodules or tumors, varying in size from that of a pea to that of a pigeon's egg. They are found on the body as a rule at the level of the trochanter, of the iliac crest, of the costal border, etc.; the nodules are rarely found on the head. Both the adult parasites and their embryos are contained in a cyst within the nodule. The males are 30 to 35 mm. long by 0.14 mm. in thickness, the females 60 to 70 mm. by 0.36 mm. The embryos measure 0.3 mm. by 0.006 to 0.007 mm. and have been found in the blood, as well as in the cyst contents.

Cutaneous myiasis is not uncommon in Tropical America and is occasionally imported to the United States. It is caused by the larva of *Dermatobia hominis* (*D. cyaniventris*). In British Honduras, the larva is known as the beef worm. The fly is found near the Southern States in Mexico. It is said that the fly seizes the female mosquito of the species *Janthiusoma lutzi* and attaches her eggs to the ventral aspect of the abdomen. When the mosquito feeds upon man, the little larva which has been held in position in the egg by its spines, slips out and pierces the skin by means of the aperture made by the mosquito bite. The larva beneath the skin produces a swelling known as the warble; it is 2 to 3 cm. and of a dark red or bluish red color. At the apex of the warble, there is a small circular aperture which increases till it is 3 to 6 mm. in diameter. When the warble is mature, the larva may be seen actively moving up and down like a jack-in-the-box, appearing and disappearing from the aperture (Castellani and Chalmers).

Another form of cutaneous myiasis is the so-called *Larva migrans*. It is caused by the larvae of several genera of flies. The presence of

the larva in the subcutaneous tissue causes a raised red line $^1/_6$ to 1 inch wide, which extends one to several inches daily and is generally sinuous.

VEGETAL PARASITES

Actinomyces bovis (p. 220) is a rare skin parasite.

Nocardia indica (Syn.: *N. madurae; Discomyces madurae; Streptothrix madurae*) is the cause of Madura foot. The "grains" found in the pus of such cases are soft, white or slightly yellowish, and have a mulberrylike surface. At the periphery of the grains radiating filaments are found; the mycelial threads are slender (1 to 1.5 micron). The fungus grows on ordinary media. It is Gram-positive, but is not acid-fast. In addition to the white or yellow maduromycoses, black and red maduromycoses are recognized, and are distinguished by the presence of black or red grains in the pus.

Trichophyton tonsurans (Syn.: *Trichomyces tonsurans; T. crateriforme; Trichophyton megalosporum endothrix*) is a cause of barber's itch (tinea sycosis) and of ring-worm of the body (tinea circinata). The organisms are found on the hairs, which are broken 2 to 4 mm. from the skin. The diseased hairs have a powdery, grayish appearance, and on pulling them out, the roots are not black, as in normal hairs. The mycelial cells are large and somewhat quadrangular, measuring 4 to 5 micra. The diagnosis is made by microscopic examination of the hairs and of scrapings and scales from the skin in potassium hydrate (10 to 40 per cent).

Trichophyton sabouraudi (Syn.: *T. acuminatum*) causes tinea capitis (ring-worm of the scalp) and also at times a variety of ring-worm of the body and barber's itch. The hairs break off even with the surface of the scalp and appear as black dots. The cells break up readily in dilute potassium hydrate (2 to 4 per cent), and have a diameter of 5 to 7 micra.

Ectotrichophyton mentagrophytes (Syn.: *Trichophyton mentagrophytes; T. asteroides; T. gypseum*) is found at times in cases of barber's itch. The mycelial spores (arthrospores) are variable in size; generally they measure 5 to 6 micra in diameter, but may not exceed 2 to 3 micra or may attain a diameter of 10 to 11 micra. The infection is observed especially in those coming in contact with horses. It is a not infrequent cause of ring-worm of the body.

Epidermophyton cruris (Syn.: *E. inguinale; Trichophyton cruris*) is the organism causing *dhobie itch*. The fungus is very abundant in

recent cases, scarce in old ones. The mycelial tubes in recent cases are generally straight, have a double contour frequently, and the segments are somewhat rectangular, their breadth being 3.5 to 4.5 micra. Branching may occur. The spores are rather large, 4 to 7 micra, roundish and generally have a double contour. They do not collect in clusters (Castellani and Chalmers).

Microsporum audouini (Syn.: *Trichophyton microsporum; Trichomyces decalvans; Sporotrichum audouini*) (Fig. 72) is a cause of one of

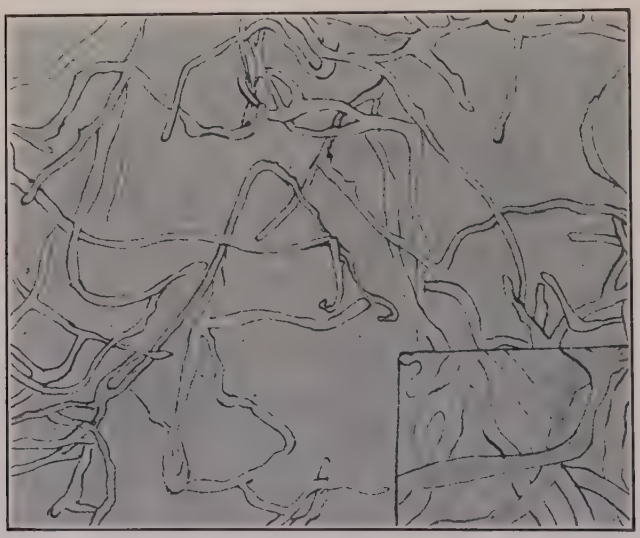

Fig. 72.—Mycelium of Microsporum Audouini in the Scales from the Scalp. Unstained. Dissociation of the scales in formic acid; washing in water; mounting in glycerin. ×260 diameters. Inset is the same preparation. ×750 diameters (after Sabouraud).

the most obstinate forms of tinea capitis. By means of its mycelial spores, the fungus forms a white opaque sheath around the affected hairs, extending 2 to 3 mm. above the scalp. The sheath is formed of a mosaic of small roundish or polyhedral spores, the diameter of which varies between 2 and 3 micra. In the interior of the hair a few mycelial filaments of the fungus may be observed (Castellani and Chalmers).

Microsporum velveticum is very similar to the preceding, and is quite common in this country.

Achorion schoenleini (Syn.: *Oidium schoenleini; Oospora porriginis*) (Fig. 73) is the cause of *favus*. The characteristic lesion is a small sulphur-yellow disc or *scutulum*, with a cuplike depression in the center, which is pierced by a hair. The fungus may also infect the glabrous parts of the body and the nails. The parasite is found in the epidermis and forms the scutula, which measure 8 to 14 mm. in diameter but may vary from 2 to 30 mm. A scutulum is crushed in alkali and examined microscopically. It is found to be composed almost entirely of the fungus. "The central portion is made up of rounded sporelike bodies of varying size without definite arrangement. Toward the periphery similar elements are seen strung out in filaments, and mixed with them

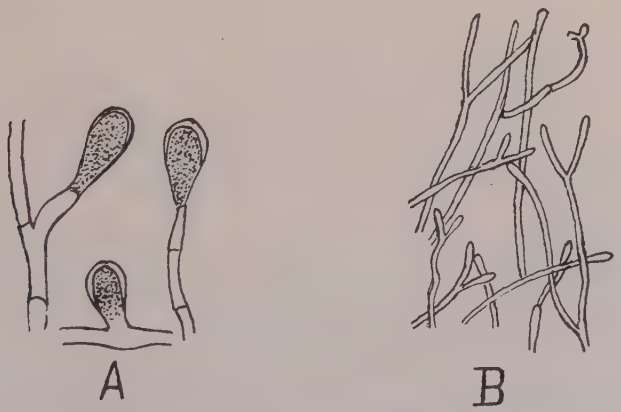

FIG. 73.—ACHORION SCHOENLEINI. *A*, so-called yellow bodies in cultures (after Bodin, from Castellani and Chalmers); *B*, claviform bodies in cultures (after Castellani and Chalmers).

hyphae of thicker elongated elements with irregular contours. Within the diseased hairs are filaments, sometimes of cubical, sometimes of elongated elements. They differ from those found in ring-worm, chiefly in that cells of different sizes and forms are found in the same case" (Zinsser).

Mycoderma dermatitis (Syn.: *Blastomyces hominis; Saccharomyces hominis; Cryptococcus gilchristi; Zymonema gilchristi; Oidium hektoeni*) probably represents a group of allied organisms rather than an individual species. In the cutaneous infections, the organisms are most frequently found in the small epidermal lesions which border the lesion. In the fresh pus they appear as round or occasionally oval, highly refrac-

tive bodies containing granules of various sizes, often vacuolated and surrounded by a hyalin capsule. They vary in diameter usually from 10 to 20 micra. Budding forms and pairs united in a figure eight can usually be found. They are more easily demonstrated if one adds 10 per cent potassium hydrate to the preparation (Fig. 58, p. 222).

Mycoderma immite (Syn.: *Coccidioides immitis; Oidium coccidioides*) is a parasite resembling *Mycoderma dermatitis,* but it does not show buds and reproduces by the formation of endospores. These appear as a mass of minute round bodies, each of which may be capsulated, within the membrane of the parent cell. The parasites vary greatly in size and some are larger than those found in blastomycosis, reaching 50 micra in diameter (Zinsser).

Melassezia furfur (Syn.: *Microsporon furfur; Sporotrichum furfur; Oidium furfur*) bears little resemblance to the organisms which cause ring-worm. It appears as septate filaments of very irregular contour 3 to 4 micra wide. They are usually unbranched but interlace, forming a meshwork in which are found masses of sporelike bodies. Most attempts to isolate the organism culturally have failed. It is the cause of *tinea versicolor.*

Sporotrichum schenki (Syn.: *Sporothrix schenki; Rhinocladium schenki*) is one of the organisms causing sporotrichosis. Morphologically, it is very similar to *S. beurmanni.*

Sporotrichum beurmanni (Syn.: *Rhinocladium beurmanni*) (Fig. 74) is also a cause of sporotrichosis. In the tissues oval, yeast-like or short bacillary forms are seen, 3 to 5 micra in length and 2 to 4 micra in breadth, either free or engulfed by the large mononuculear phagocytes (Castellani and Chalmers). According to Zinsser, it is difficult and often impossible to demonstrate the parasites by direct examination of the pus from the sinus. The examination is facilitated by clearing the pus with 40 per cent sodium hydrate or by staining with thionin or other basic stains. The organisms are Gram-positive. Cultures are often required to demonstrate them (Fig. 74).

FIG. 74.—SPOROTRICHUM BEURMANNI (after Gougerot, from Castellani and Chalmers).

According to Zinsser, the best method for the diagnosis of sporotrichosis consists in cleansing the skin over one of the closed lesions

with tincture of iodin and aspirating pus from the abscess. Tubes of Sabouraud's test medium or of 4 per cent glucose agar should be heavily inoculated on the surface and incubated at room temperature. The colonies appear in four days or more. (For the cultural characteristics, the reader is referred to works on bacteriology.)

CHAPTER VIII

THE BLOOD

Obtaining Blood for Examination.—Blood for counts, etc., is obtained most conveniently from the lobe of the ear or the ball of the finger. The ear is, on the whole, more satisfactory than the finger. It is easily accessible, the flow of blood is as good, and it is much less sensitive to pain than the finger. There is also less likelihood of infection of the small wound through contact with dirty objects. For counts or hemoglobin determinations, blood should *not* be drawn from a part of the body which is cyanotic, because concentration of the blood may occur, producing results which are misleading (too high).

Blood Stickers.—A number of satisfactory blood stickers are on the market, and require no special description. In default of these a Hagedorn needle may be used. Bass [1] has recently described a simple arrangement consisting of a straight surgical needle mounted in a cork. When not in use, the needle is carried in a small vial filled with alcohol. A sharp steel pen, one of whose prongs has been broken off, may be employed as a sticker. The sticker should always be perfectly clean, in addition to being sterile. Dried blood on the point, even a very small amount of it, makes a sharp instrument seem dull.

Method.—The skin of the ear or finger and the sticker are cleaned with alcohol or ether, which is allowed to evaporate completely. The skin is then pierced. The point of the sticker should be held close to the skin and pushed in rather quickly; beginners frequently make a sudden stab at the part from a distance of several inches, either missing the skin entirely or producing an unnecessarily deep wound. The wound must be such that the blood flows freely from it; squeezing the tissues to obtain blood is not permissible, since the blood is thereby diluted with lymph.

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[1] Bass, C. C. "A practical, inexpensive, aseptic blood-sticker." *Med. Record*, 1910, LXXVIII, 538.

COUNTING THE BLOOD CORPUSCLES

The Hemocytometer.—In blood counting, the standard instrument in universal use is the hemocytometer of Thoma (Fig. 75). The instrument, as originally designed by Thoma, was not satisfactory for the enumeration of the leukocytes, and, as a result of this, numerous modifications of the Thoma ruling of the counting chamber have been brought forward.[2] The change consists in an increase of the ruled area from 1 sq. mm. to 9 sq. mm. Neubauer, Türk, Zappert-Ewing, and others have devised rulings, which are designated by their names. The writer prefers the Neubauer ruling (Fig. 76). In all the modifications the

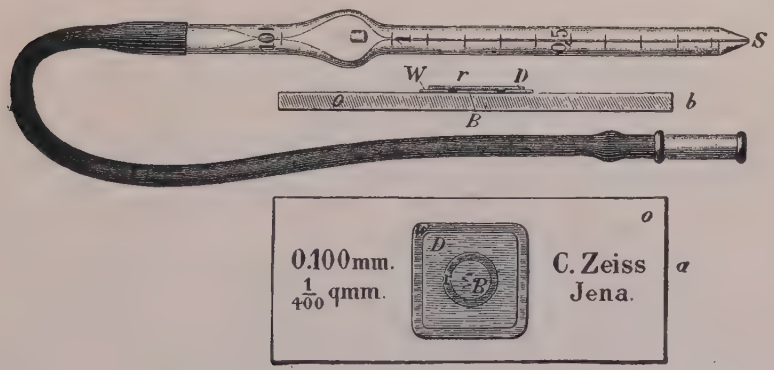

FIG. 75.—THE THOMA HEMOCYTOMETER.

central square millimeter retains the original ruling of Thoma. The additional eight square millimeters surround it, and are solely for greater accuracy and convenience in counting the white corpuscles.

THE RULING OF THE COUNTING CHAMBER.—There is, then, a ruled area 3 mm. on a side or 9 sq. mm. (Fig. 76). This area is divided into nine large squares, each of which is 1 mm. on a side or 1 sq. mm. The *central* square millimeter, which is used for counting the erythrocytes, is subdivided into 400 small squares, each of which is $\frac{1}{20}$ mm. on

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[2] The writer learns from dealers in laboratory supplies that they are forced to keep the original counting chamber in stock, since physicians specify "Thoma-Zeiss" in ordering. This is doubtless due to the fact that the purchasers are unfamiliar with the much more practical rulings—modifications of the Thoma—which are mentioned above.

a side, and has, therefore, an area of $\frac{1}{400}$ sq. mm.[3] By means of double lines these smallest squares are grouped into blocks of twenty-five, a convenient unit to employ in counting. The ruling of the *remaining eight large squares* (1 sq. mm. each) varies according to the design selected. For the leukocyte count the entire nine square millimeters may be used.

Construction of the Counting Chamber (Fig. 75).—The ruling is on a glass disc (*B*) which is mounted on a heavy glass slide (*o*). The disc is surrounded by a glass table (*W*), also attached to the slide, the surface of which is exactly one-tenth (0.1) of a millimeter above that

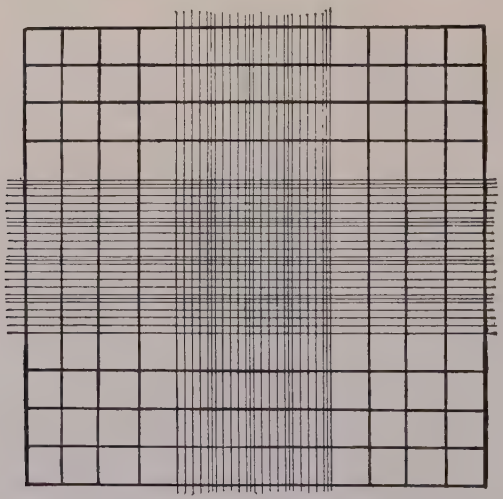

Fig. 76.—The Neubauer Ruling of the Hemocytometer.

of the ruled disc. A moat (*r*) about 2 mm. wide separates the disc from the table. When the cover glass (*D*), which is supplied with the apparatus, is placed upon the glass table, it thus forms a space between its under surface and the surface of the ruled disc, which is 0.1 mm. deep.

The modification of the *Bürker counting chamber*[4] (Fig. 77), which is now quite extensively used, possesses several advantages over the older

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[3] This is always indicated on the glass slide of the counting chamber— $\frac{1}{400}$ sq. mm. The depth is also given— $\frac{1}{10}$ mm. deep. (Fig. 77.)

[4] Bürker, K. "Erfahrungen mit den neuen Zählkammer, nebst einer weiteren Verbesserung derselben." *Arch. f. d. ges. Physiol.*, 1907, CXVIII, 460.

design of Thoma. With the Bürker chamber, the cover glass is placed in position, and then the diluted blood is allowed to run under the cover glass from the tip of the pipette by capillary attraction. The same precautions, described in connection with the Thoma chamber, as to cleanliness of the chamber and cover glass, and shaking the pipettes to secure a uniform suspension of the corpuscles, must, of course, be followed.

Bürker calls attention to the following technical points which should be observed: (*a*) To avoid bubbles, the cover glass and chamber must

Fig. 77.—The Bürker Counting Chamber with Single and Double Neubauer Rulings.

be carefully cleaned. Furthermore, in using pipettes having an angle at their tips, bubbles are prevented with difficulty; the tip should be rounded off with emery paper. (*b*) The drop of blood, which flows under the cover glass, should not be so large as to overflow into the gutter. Counting chambers should not be purchased, in which the gutter between the two ruled surfaces is less than 2 mm. wide and the lateral gutters less than 1.5 mm. wide. (*c*) After allowing the red cells to settle for at least three minutes, the evenness of distribution should be determined before proceeding with the count. Bürker advises that this be accomplished in the following manner: The counting chamber is placed on the stage of the microscope, illuminated by the mirror with

the diaphragm opened wide. By viewing the counting surface obliquely with the unaided eye, one sees a film or veil formed by the erythrocytes. Irregularities in the distribution of the cells are shown by variations in the density of the film. When such irregularities are visible, the chamber must be refilled. In counting the leukocytes, this procedure is not applicable, for the cells are too few. Microscopic examination with low power must be made.

It is advisable, in purchasing, to select a chamber with double Neubauer ruling. The one may then be used for the red count, the other for the white; filling the chamber in this way conserves time.

The Diluting Pipettes.—Since whole blood is much too thick to permit direct enumeration of its corpuscular elements, pipettes with which accurate dilutions of the blood can be made are required. Two pipettes are furnished with the complete hemocytometer, one for the red cells, the other for the white. Each consists (Fig. 75) of a capillary tube, which opens into a bulb containing a glass pearl. The capillary tube is divided into 10 equal parts. In the red pipette the bulb, when filled to the line on its upper outlet (marked 101), holds one hundred times the contents of the ten divisions of the capillary tube. It is, therefore, possible to obtain ten different dilutions of blood, if desired. Practically, only two dilutions are employed—1:200 and 1:100.

With the *white pipette* lower dilutions are made. The bulb usually contains either ten or twenty times the content of the capillary tube. Dilutions of 1:10, 1:20, etc., are generally made.

Procedure in Counting the Erythrocytes

1. **Diluting Fluids.**—The requirement for the diluting fluid is that it preserves well the red corpuscles. Numerous formulæ have been elaborated. Among the better known of these are the following:

a. Hayem's solution:

Bichlorid of mercury	0.5 gm.
Sodium chlorid	1.0 gm.
Sodium sulphate	5.0 gm.
Distilled water	200.0 c.c.

Dissolve and preserve in a tightly stoppered bottle.

This is the most satisfactory diluting fluid. It keeps indefinitely, and no organisms grow in it. The red cells settle evenly in it.

b. Physiological salt solution:

Sodium chlorid	0.85	gm.
Distilled water	100.0	c.c.

Dissolve.

The red cells settle slowly and often unevenly in salt solution.

c. Toisson's fluid:

Sodium sulphate	8.0	gm.
Sodium chlorid	1.0	gm.
Glycerin (neutral)	30.0	c.c.
Distilled water	160.0	c.c.
Methyl violet	q. s.	

Dissolve. (The methyl violet is added in minute amount [25 to 30 mg.], just enough to color the fluid, which should remain clear and transparent.)

Toisson's fluid is not stable and must be filtered before using. Low forms of vegetable life luxuriate in it, and it is, on the whole, an unsatisfactory fluid, its only advantage—which is usually negligible—being that leukocytic and other nuclei are stained.

2. **Filling the Pipette.**—The first essential is to have the blood flowing freely and to obtain a fresh drop. If it is necessary to squeeze the ear to obtain the blood, the latter will be diluted with tissue lymph, making the count too low; if the drop is not perfectly fresh, clotting will have begun, so that a uniform suspension of the cells cannot be secured. The blood is sucked cautiously into the capillary tube to the line marked 0.5. (With anemias of 2,500,000 cells or less, the blood is more conveniently drawn up to the mark 1 to secure a lower dilution, i. e., 1:100.) If the blood is accidentally sucked above the line, it may be lowered by drawing the finger across the tip of the pipette, *provided* the column of blood has not passed more than 1 mm. above the line; if it has extended farther, the blood adhering to the wall of the tube will be sufficient to introduce a serious error in the dilution. In the latter case the blood should be drawn up to the next line on the capillary tube, 0.6, quickly, and a corresponding correction in dilution calculated. Bubbles of air in the column of blood must, of course, be avoided. After the tube has been accurately filled with blood, the end of the pipette is wiped free of blood, and is then plunged into the diluting fluid, which is sucked up to the line marked 101. While the diluting fluid is being

drawn up, the pipette, held between thumb and fingers, is revolved to keep the glass pearl within the bulb in motion, both for the purpose of mixing the blood and diluting fluid and also to prevent bubbles adhering to the pearl, which would render the dilution inaccurate. The filling of the capillary tube and the subsequent dilution of the blood require rapid manipulation to prevent clotting. When the fluid reaches the line 101, the mouthpiece of the pipette is occluded with the tongue. The finger is then placed over the tip of the capillary tube, the thumb grasping the other end of the pipette. An even and uniform suspension of the erythrocytes is now secured by shaking the pipette, held *horizontally,* for at least two minutes. After the shaking is completed, several drops are blown out of the pipette to thoroughly empty the capillary tube (which contained only diluting fluid), and the counting chamber is then filled. Since the red corpuscles settle quite rapidly, the contents of the pipette must be perfectly mixed by shaking each time before filling the counting chamber.

3. **Filling the Counting Chamber.**—The counting chamber and cover glass must be perfectly clean and free from dust. A drop of the diluted blood is placed at one side of the ruled disc; the cover glass, used as a lever, is gradually lowered onto the drop, the edge of the glass table serving as the fulcrum. As the cover glass comes in contact with the drop, and the latter spreads over the surface of the ruled disc, there will be a tendency for one or more bubbles to form. By alternately raising and lowering the cover glass the fluid will spread evenly, and bubbles can be avoided. The size of the drop of diluted blood which is taken is important, and is learned by practice. It should be just large enough to cover the surface of the ruled disc, when the cover glass is applied. If it is so large that much of the fluid runs into the moat, or that any fluid is found between the cover glass and glass table, the counting chamber must be cleaned and refilled. Before proceeding to count the erythrocytes, two conditions must be fulfilled in addition to those already given: (1) Newton's rings (prismatic colors) must be visible between the cover glass and glass table when looked at obliquely toward the light, since this proves that the two surfaces are in close apposition and are not separated by a particle of dust, which would deepen the chamber; and (2) inspection under low magnification should show no clots and no gross inequalities in the distribution of the red cells over the ruled surface. These conditions being fulfilled, after allowing the erythrocytes to settle in the fluid two or three minutes so that

all of them will be in the same focus, the enumeration of the cells is proceeded with.

Filling the Bürker counting chamber is simpler (see p. 251).

In making the count it is advisable, if the worker is inexperienced, to use a high magnification (Leitz ocular 1, objective 6, or corresponding values for other makes of microscope), which will include a single block of twenty-five small squares. Ordinarily, however, a lower magnification is sufficient, such, for example, as one obtains with Leitz ocular 3, objective 3, with the tube drawn out to its full length.

4. **The enumeration of the cells** may be made in an almost endless number of ways. A good method is as follows: The cells in 100 small squares are counted. This is accomplished by counting separately four blocks of twenty-five small squares, the blocks used being those at the corners of the ruled area. Beginning at the upper left corner of a block of twenty-five small squares, the count is made from left to right in the upper tier of five small squares, then from right to left in the second tier, and so on, until the entire block of twenty-five has been covered. The *cells touching the line* on two adjacent sides of a square are counted, while those on the line of the two remaining sides are disregarded. The total count for each block of twenty-five small squares is set down. When all four blocks have been counted, the difference between the highest and lowest total count should not exceed 25 cells. If this limit is exceeded, the distribution of the cells in the counting chamber has been so uneven as to make the results untrustworthy.[5]

5. **Calculation of the Result.**—The calculation is simple. Since each small square is $\frac{1}{20}$ mm. on a side, and the counting chamber is $\frac{1}{10}$ mm. deep, the cubic content of one small square is $\frac{1}{20} \times \frac{1}{20} \times \frac{1}{10}$ or $\frac{1}{4000}$ c. mm. As 100 such squares have been counted, it follows that the sum of all the cells counted is the number of cells contained in $\frac{100}{4000}$ or $\frac{1}{40}$ c. mm. of *diluted* blood. Since the dilution used was 1:200, the *total* number of cells counted in the 100 small squares must be multiplied by 40 and by 200, or by 8,000, to obtain the number of cells in 1 c. mm. of *undiluted* blood, the desired result.

Example.—In the 100 small squares, let us assume that the total number of cells is 620. To obtain the total number of cells in 1 c. mm. of *undiluted* blood, this number must be multiplied by 40 (i. e., $\frac{1}{40}$

[5] Following Emerson, students are required to fulfill these conditions and to make counts on two successive days, which shall not differ by more than 200,000 cells per c. mm., the limit of error of the method.

c. mm. diluted blood) and by 200 (dilution 1:200), or multiply by 8,000. Thus:

(a) $620 \times 40 \times 200 = 4{,}960{,}000$. Or,
(b) $620 \times 8{,}000 = 4{,}960{,}000$.

The *normal count* usually given for healthy adults is: for males, 5,000,000; for females, 4,500,000 cells per c. mm. These figures represent averages. The red count of normal adults is not infrequently as high as 6,000,000. (For pathological changes in the red count, see pp. 314–320.)

6. **Cleaning the Apparatus.**—(a) *The Counting Chamber.*—The counting chamber should be cleaned with *water only.* After thorough rinsing, it is wiped dry with a soft, clean cloth. The diluted blood should never be permitted to dry in the counting chamber. Alcohol, ether, or similar solvent should not be employed, as it may dissolve the cement, by which the ruled disc and the glass table surrounding it are fastened to the slide. (In case this accident happens, all the parts may be returned to the maker, for it is possible at times to repair the damage.)

(b) *The Pipettes.*—The pipettes should be cleaned immediately on completion of the count. If this is impossible, they should at least be emptied and refilled with water, until it is convenient to clean them. If allowed to stand, the diluted blood drying in the tip of the capillary may occlude it. The successive steps are as follows:

(1) The pipette is emptied of its contents.

(2) Distilled water or clear tap water is drawn into the pipette. After emptying, fill it with—

(3) Ethyl alcohol, 95 per cent. Shake the pipette so that the water adhering to the pearl is mixed with the alcohol, place the rubber tubing over the tip of the capillary tube, and blow the alcohol through.

(4) Fill the pipette with ether, and shake again. Remove the rubber tubing, and allow the ether to run out by inverting the pipette.

(5) Aspirate air through the pipette till the walls of the bulb are dry and the glass bead rolls freely as the pipette is rotated. If the bead sticks to the wall, it means that there is still moisture remaining, or that the pipette is not clean.

A suction pump is a great convenience in cleaning the pipettes. As a substitute for the pump, a stiff-walled rubber bulb may be employed.

If blood clots in the capillary tube, it should be removed as soon as possible by means of a horse hair. For this purpose hairs from the

tail or mane are washed in water and alcohol and kept in the latter. Wire should never be used, as it may scratch the glass, and there is also great danger of chipping the end of the pipette. At times, if the clot has become very firm or dry, it is necessary to place the pipette in a test tube with nitric acid. When a film of coagulated albumin forms over the inner surface of the bulb, the pipette may be filled with nitric acid and set aside for several hours.

Counting the Leukocytes

1. **Diluting Fluid.**—For counting the white cells of the blood it is necessary to have a fluid which will render the erythrocytes invisible and cause the leukocytic nuclei to stand out prominently. Dilute acetic acid is universally employed for this purpose. It is used in about 1 per cent strength. The solution is quickly prepared by adding two drops of glacial acetic acid to 10 c.c. of distilled water. The dilute acid should be prepared freshly each day; yeast cells grow in it, if it is kept, and may lead to confusion, since single cells resemble the nuclei of lymphocytes, while several cells are not very unlike a polymorphous nucleus. It is convenient to have a small, wide-mouthed bottle with a file-scratch indicating 10 c.c. for preparing the dilute acetic acid.

2. **Filling the Pipette.**—The capillary tube of the white pipette is larger in caliber than that of the red pipette, and, therefore, a larger drop of blood will be required to fill it. The blood is sucked up to the mark 0.5, as a rule; the blood on the end of the pipette is wiped off, and the tip immediately plunged into the diluting fluid, which is sucked up to the mark 11 (or 21 in the case of the larger white pipettes). The pipette must be rotated more jerkily and the fluid sucked in more slowly than with the red pipette, to avoid the air bubble which so often clings to the glass pearl. After the pipette is filled the end is occluded, and the pipette, held horizontally, is shaken at least two minutes. In short, all the precautions requisite to a proper filling of the red pipette (to which the reader is referred) apply with equal force here. Bubbles in the column of blood or in the bulb of the pipette ruin the preparation. The column of blood must be drawn quickly and accurately to the desired mark.

When the number of leukocytes is greatly increased, as is the case in extreme leukocytoses and usually in leukemia, it is often more convenient to use the red pipette for the leukocyte count in order to obtain a greater dilution.

3. **Filling the Counting Chamber.**—The counting chamber is filled in the manner described under the red cell count. Since the capillary tube of the white pipette is wider, the diluted blood flows out of it more rapidly, and the size of the drop is less easily controlled. A drop of the right size, Newton's rings, and an even distribution of the cells over the ruled area are essential.

The magnification employed should be the same as that used in counting the erythrocytes (q. v.). Work is much more rapid with the lower power.

4. **The Enumeration of the Leukocytes.**—After the cells have settled until all are in the same focus (usually in two or three minutes), the leukocytes, whose nuclei stand out as refractive bodies, are counted in one square millimeter at a time, including the cells which touch the line on two sides of the square only. Nine or ten square millimeters are counted—nine in one preparation, or five in each of two preparations. The procedure is the same as that used in counting the red corpuscles, the only difference being the larger unit employed, 1 sq. mm. The difference between maximal and minimal total count for 1 sq. mm. should not exceed 8 cells.

The *normal leukocyte count* of adults lies between 5,000 and 10,000 cells per c. mm.—rarely 12,000 cells. (For pathological alterations in the leukocyte count, see pp. 321–329.)

If the diluting fluid is not freshly prepared, *yeast cells* may be counted and lead to serious error. A second source of error is the presence of a considerable number of *nucleated red cells* in the blood. Ordinarily erythroblasts are detected only when the stained blood is simultaneously examined; and, as they are numerous only in marked pathological states of the blood, they seldom escape notice. They cannot well be separated from the leukocytes in the counting chamber, so that the count obtained is the sum total of all nucleated cells in the blood—both red and white. The number of erythroblasts is determined by making a differential count of the stained blood and noting the relative number of nucleated red cells as compared with the leukocytes. From the proportion of the two kinds of cells found, the correction of the white count is made. Thus, if the leukocyte count were 20,000, and differential count showed 125 nucleated reds to 500 leukocytes, the relative frequency of the two would be as 1:4. Therefore, there were present in one cubic millimeter of blood 5,000 nucleated red cells. Since these were included with the leukocytes in the total count, the latter must

be corrected by deducting the nucleated reds. The leukocyte count thus becomes 15,000 instead of 20,000.

5. **Calculation of the Result.**—Let us assume that the dilution employed was 1:20, and that nine square millimeters were counted. Each square millimeter has a cubic content of 0.1 c. mm., since the counting chamber is 0.1 mm. deep. The sum of the leukocytes in the nine large squares divided by 9 gives the average number of cells in 0.1 c. mm. of *diluted* blood. To obtain the number of cells in 1 c. mm. of *undiluted* blood, this number is multiplied by 10 and by 20 (dilution 1:20), or by 200.

Example.—Let us assume that the total number of leukocytes counted in the nine large squares is 270. This number, divided by 9 (the number of squares counted), gives 30, the average number of cells in one square (i. e., in 0.1 c. mm.). This number, multiplied by 10, gives the number of cells in 1 c. mm. of diluted blood. Multiplying again by 20 (dilution 1:20) gives the total number of cells per cubic millimeter of undiluted blood. Thus:

(*a*) $30 \times 10 \times 20 = 6{,}000$. Or,
(*b*) $30 \times 200 = 6{,}000$.

Counting the Eosinophilic Leukocytes

The number of eosinophilic cells is usually arrived at by making a total count of the leukocytes in the ordinary way and, at the same time, preparing stained specimens. By finding the percentage of eosinophiles in a differential count, the absolute number of cells per cubic millimeter may then be calculated.

Dunger[6] has devised a method by which the absolute number of eosinophiles per c. mm. may be determined directly.

Dunger's Method.—The formula of the diluting fluid is:

1 per cent aqueous solution of eosin	10.0 c.c.
Acetone	10.0 c.c.
Distilled water	90.0 c.c.

The solution must be preserved in a tightly corked bottle to prevent evaporation of the acetone, and is then quite stable.

[6] Dunger, R. "Eine einfache Methode der Zählung der eosinophilen Leukocyten und der praktische Wert dieser Untersuchung." *München. med. Wchnschr.*, 1910, LVII, 1942.

A 1:10 dilution of the blood is made in the white pipette, and the mixture is thoroughly shaken three to five minutes. After blowing out the contents of the capillary tube, a drop is placed in the counting chamber (ruled for leukocyte count, i. e., 9 sq. mm.). Only the eosinophile cells are well seen; they appear as small, pink bodies. With a magnification of 120 to 150 diameters they are readily seen. The entire nine square millimeters of the chamber are counted. Ordinarily 9 to 18 eosinophile cells are found in this area; this corresponds to about 100 to 200 eosinophilic leukocytes per c. mm. The calculation of the total number of cells is made in the way described for counting the leukocytes (p. 259). After a little practice an increase in the number of these cells is recognized at a glance. By making a leukocyte count in the usual manner simultaneously, the *percentage* of eosinophiles may be determined.

Counting the Blood Platelets

Several methods have been proposed for counting the platelets. The *indirect method* has given fair results, i. e., making a count of the erythrocytes in the usual way and at the same time determining the relative number of platelets as compared to the red cells in a fresh specimen of blood. The number of platelets is then calculated. *Direct methods* of counting platelets have been attempted; the only one which appears to give reliable results is that of Wright and Kinnicutt.

Method of Wright and Kinnicutt.[7]—*The diluting fluid:*

Solution 1:

"Brilliant cresyl blue"	1.0 gm.
Distilled water	300.0 c.c.

Dissolve. Keep on ice to prevent the growth of yeasts.

Solution 2:

Potassium cyanid	1.0 gm.
Distilled water	1,400.0 c.c.

Method.—"The blood is mixed with the diluting fluid in the proportion of 1:100 by means of the pipette used for counting red blood corpuscles, and the counting is done in the ordinary counting chamber

[7] Wright, J. H., and Kinnicutt, R. "A new method of counting the blood platelets for clinical purposes and some of the results obtained with it." *Jour. A. M. A.*, 1911, LVI, 1457.

with a high-power dry objective. In order to render the platelets more clearly visible, the specially thin cover glass of Zeiss, with central excavation, is used (cover glass No. 146, Zeiss catalogue).[8] The diluting fluid consists of *two parts* of the aqueous solution of 'brilliant cresyl blue' (solution 1), and *three parts* of the aqueous solution of potassium cyanid (solution 2). These two solutions must be kept in separate bottles and mixed and filtered immediately before using. Of course, the pipette should be well shaken after withdrawing the sample for counting. After the counting chamber is filled, it is left at rest for ten or fifteen minutes, in order that the blood platelets may settle to the bottom of the chamber and be more easily and accurately counted.

"The platelets appear as sharply outlined, round or oval or elongated, lilac-colored bodies, some of which form a part of small spheres or globules of hyalin, unstained substance.

"The red cells are decolorized and appear only as 'shadows,' so that they do not obscure the platelets. The nuclei of the white cells are stained a dark blue, the protoplasm light blue. If the technic is correct, there should be no precipitate in the preparation.

"The cresyl blue solution is permanent, but should be kept on ice in order to prevent the growth of yeasts. The cyanid solution should be made up at least every ten days. It is, of course, necessary that the solution be made from pure potassium cyanid, which has not undergone decomposition. As already stated, the two solutions must be mixed and filtered immediately before using, because after filtration, if the mixture is allowed to stand exposed to the air for a short time, a precipitate will form in it. After the diluting fluid has been mixed with the blood in the pipette, however, no precipitate forms, and, as the platelets do not quickly break up in the mixture, the counting may be done after some hours, if necessary. For example, a count immediately after filling the pipette was 258,000 and another count from the same filling of the pipette made eighteen hours later was 253,000.

"A proper technic yields a remarkably even distribution of the platelets in the chamber. For all practical purposes, the counting of the platelets in 100 small squares is sufficient, but for greater accuracy all 400 small squares should be counted, or 200 small squares in each of two fillings of the chamber."

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[8] This special cover glass is, however, unnecessary, if one has the usual thin cover glass, which permits the use of the high-power dry objective.

With their method Wright and Kinnicutt find that the *platelet count* of normal adults varies between 226,000 and 367,000 per cubic millimeter, the general average being 297,000.

HEMOGLOBIN DETERMINATIONS

Many methods for the determination of the amount or percentage of hemoglobin have been brought forward. For a description of all of them the reader is referred to the textbooks of hematology.

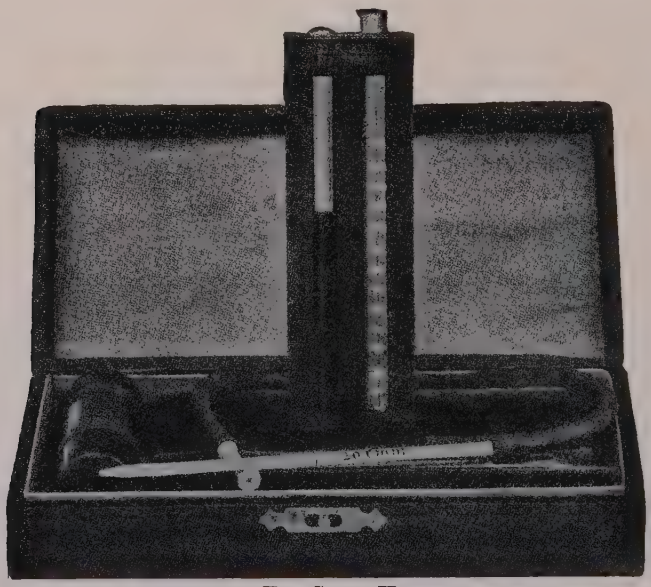

FIG. 78.—THE SAHLI HEMOMETER.

1. **Tallqvist** devised a *color scale,* which has been widely used. It consists of a series of ten shades of red, intended to represent the color intensity of hemoglobin from 10 per cent to 100 per cent. Each color is perforated. A drop of blood is collected on a filter paper, supplied in the book containing the scale, and, as soon as the gloss has disappeared from the drop, it is placed under the perforation in one of the red strips. It is moved until the color of the drop of blood corresponds with one of the shades of red. This represents the hemoglobin percentage of the blood. With the Tallqvist scale it is possible, perhaps, to make a more accurate guess as to the percentage of hemoglobin than without it. It

is well recognized that the scale is very inexact. In fact, the color scales in separate books do not always match. When the blood is hydremic, the plasma runs beyond the corpuscles, which are concentrated at the center of the drop, introducing an additional error in the very cases where more exact results are desirable. If a hemoglobin determination is indicated a little more time should be spent than is required with the Tallqvist scale, in order to obtain a result of some value.

2. **Sahli's Hemometer.**—Sahli's hemometer is a modification of the old Gower instrument. It consists (Fig. 78) of one tube containing the standard solution and a second tube of the *same caliber* graduated from 0 to 140, each division representing 20 c. mm. The tubes are placed in a hard rubber stand, which has an opaque glass back. A pipette with a line representing 20 c. mm. is supplied with the instrument. The standard solution is one of acid hematin, prepared as follows:[9]

Blood	1 part
$\frac{N}{10}$ hydrochloric acid	10 parts
Distilled water to	50 parts
Mix and add—	
Glycerin	50 parts

The hemoglobin is converted into acid hematin, which does not go into solution, but is in a very fine state of suspension. Therefore, the hematin settles slowly, when the instrument is not in use, and for this reason a glass pearl is placed in the tube to facilitate mixing the standard fluid, which should be done each time immediately before using. Sahli[10] obtains the blood for the standard solution from young adult males having a high red cell count. This explains the fact that normal blood seldom shows more than 90 per cent of hemoglobin with the Sahli hemometer, when a new instrument is employed. In the course of time the standard solution fades. If the tube is protected from the light when the instrument is not in use, however, it may be kept as long as two years or more without serious deterioration.

The standard solution should be made from bloods of normal adults

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[9] Hastings, T. W. "The estimation of hemoglobin-content of blood with modern instruments." *Jour. A. M. A.*, 1907, XLVIII, 1749.

[10] Sahli, H. *Diagnostic Methods* (1st Amer. ed.), Phila. and London, 1905, p. 620.

having 5,000,000 red cells per 1 c.mm. with an oxygen combining power of 20.9 c.c. per 100 c.c. of blood. This corresponds to 15.6 gm. of hemoglobin per 100 c.c., which is the *normal* amount of *hemoglobin* for both men and women, as Haden [11] has shown.

It is more satisfactory to prepare the standard solution from blood with a 5,000,000 count, checking it with other similar bloods. By doing this the standard tube may be refilled, say, every six or twelve months, doing away with the necessity of corrections. The values obtained are then safe for use in determination of the color index.

It is very important that the standard tube and the graduated tube have the same diameter.[11a] If unequal, it is clear that the results obtained will be without value.

Method.—The graduated tube is filled accurately to the mark 10 with tenth normal hydrochloric acid. The pipette is now filled with blood exactly to the line marked 20 c. mm. The blood is quickly discharged into the acid in the graduated tube, and the pipette is rinsed two or three times with the acid to remove that which adheres to the wall of the pipette. The graduated tube is immediately shaken to secure a uniform suspension of the blood before clotting will have begun. The blood quickly becomes dark brown in color from the conversion of the hemoglobin into acid hematin. The mixture of blood and acid is allowed to stand exactly one minute, and is then diluted with water, until its color matches that of the standard solution. Daylight or artificial light may be used, since the tubes contain the same substance.

When the graduated tube is inverted to secure thorough mixing, care should be exercised that none of the fluid adheres to the finger, for enough may be removed in this way to cause a considerable lowering of the reading. When comparing the colors, it is well to rotate the graduated tube until the lines on it are not visible. When the colors have been accurately matched, the instrument is set aside for a couple of minutes to allow the fluid in the graduated tube adhering to the wall to run down. The height of the column is then read. This gives the

[11] Haden, R. L. "The normal hemoglobin standard." *Jour. A. M. A.*, 1922, LXXIX, 1496. See also Terrell, E. H. "On the colorimetric determination of hemoglobin with especial reference to the production of stable standards." *J. Biol. Chem.*, 1922, LIII, 179.

[11a] Tubes from uniform tubing have been made for several years for the writer by Eimer and Amend, Third Ave., New York City. The standard tube is made in the form of the usual test tube. When filled with the standard solution it may be sealed in the flame, though it is more convenient to use a paraffined cork, as in this way it may be refilled an indefinite number of times.

hemoglobin percentage, the color of the standard fluid being considered as 100 per cent.

In cases where the hemoglobin is extremely low it is difficult to obtain satisfactory readings. In such case 40 c. mm. of blood may be added to the acid. The final result is then divided by 2.

Stäubli [12] has made a critical study of this method of determining hemoglobin, using the Sahli hemometer and the Autenrieth-Königsberger colorimeter. He finds that there is a progressive darkening of the acid hematin formed by mixing the blood with the tenth-normal acid. The darkening is most rapid in the first few minutes after the mixture is prepared; plotting the values obtained, he found that the curve is a parabola. He has demonstrated that it is important to use tenth-normal hydrochloric acid, not an approximate dilution, and to measure it into the graduated tube accurately, for the rapidity of darkening of the blood is directly proportional to the quantity and concentration of acid. The blood-acid mixture should be allowed to stand exactly one minute, as Sahli recommends, and should then be quickly diluted with water, which inhibits the effect of the acid. Stäubli suggests that a better method of procedure with the Sahli hemometer is as follows: The blood-acid mixture is diluted at once with tenth-normal hydrochloric acid, until the color is approximately that of the standard tube; then wait for ten minutes [13] to make the final comparison. The final dilution, which will require only a few drops, may be made either with water or with the acid. This technic in his hands has yielded uniform results with all bloods.

Whichever method is followed, it is absolutely essential that it be adhered to strictly in order to obtain comparable results.

Aside from variations in the standard fluids and possible lack of uniformity in diameter of the tubes, it is probable that Stäubli's findings explain to a great extent the anomalous results which many workers have obtained with this instrument.

Cleaning the Hemometer.—The graduated tube is rinsed with water. The pipette is rinsed first with water, then with alcohol and with ether. Finally, air is aspirated through it to dry the pipette.

3. **The Fleischl-Miescher Hemoglobinometer.**—This instrument is

[12] Stäubli, C. "Zur Ausführung der Hämoglobinbestimmung. (Unter Umwandlung des Hämoglobins in salzsaures Hämatin.)" *München. med. Wchnschr.*, 1911, LVIII, 2429.

[13] Ten minutes is the time interval selected, since it was found that the darkening which occurs beyond this interval is slight.

generally considered to be the most accurate for the determination of hemoglobin. It is not well adapted to general use, since it is expensive and requires a dark room for making the readings.

The instrument (Fig. 79) consists of a standard, on which a wedge of red glass is mounted, cells of 12 and 15 mm. depth, and a mixing pipette. The *pipette* is similar to those used with the hemocytometer. The markings on its capillary tube, $^1/_2$, $^2/_3$, and 1, permit of dilutions of the blood of 1:400, 1:300, and 1:200 respectively. Sodium carbonate, 0.1 per cent aqueous solution, is used as the *diluting fluid.* The *cells,*

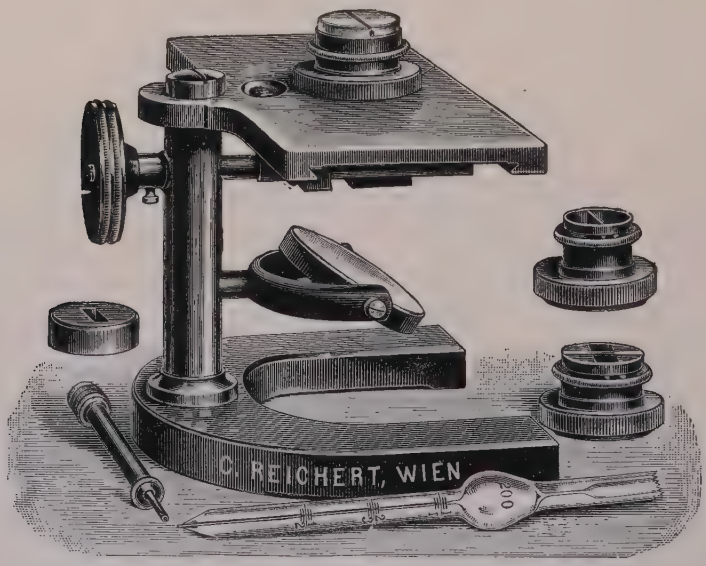

Fig. 79.—The Fleischl-Miescher Hemoglobinometer.

12 and 15 mm. deep respectively, are divided into two equal parts, one of which is filled with water, the other with the diluted blood. Each compartment should be filled until the surface of the fluid is convex above the upper level of the cell. The cell is then sealed by a glass disc, care being exercised to avoid bubbles in the fluids. Finally, a metal cap is placed over the glass disc. In the cap there is a slit, which should be so placed that its long axis is at a right angle to the partition dividing the cell. The cell is now placed on the stand, so that the compartment containing water is directly above the red glass wedge. Candlelight furnishes the most satisfactory illumination; it must be used in a dark

room. All direct rays of light are cut off from the eye of the examiner, either with a large cone or by placing the instrument in a box with one side—at which the operator stands—open and a small hole cut in the opposite side near the bottom for illumination of the reflector. The colored prism is now moved until the shades of red in the two divisions of the cell are alike. The reading is made on the scale and recorded. Ten such readings should be made, and the average of them taken. If the cell 15 mm. deep has been used, the glass disc is removed and the diluted blood sucked back into the pipette. The 12-mm. cell is then filled, and ten readings are made with it, and the average taken. The latter should be four-fifths of the reading obtained with the 15-mm. cell, and should not vary by more than 2 per cent. The readings obtained do *not* represent hemoglobin percentages. They are to be used in connection with the table found in a pamphlet supplied with each instrument. (As the instruments are separately standardized, the tables often differ, and, therefore, cannot be used interchangeably.) From the table the hemoglobin in *grams per 100 c.c. of blood* is calculated according to the directions in the pamphlet. All values are to be reduced to a dilution of 1:300 with the 15-mm. cell. (For normal blood the 1:300 dilution is usually employed. With anemic blood use a dilution of 1:200, and with plethoric 1:400.)

Haden [14] finds 15.6 gm. hemoglobin per 100 c.c. normal. This corresponds to blood with an oxygen capacity of 20.9 c.c. per 100 c.c. of blood.

In leukemia or with extreme leukocytoses readings may be difficult, because of the opacity produced by the white cells. The leukocytes may be removed by centrifugalizing the diluted blood before filling the cell.

Cleaning the Hemoglobinometer.—The pipette is cleaned in the same manner as the counting pipettes (q. v.). The cells should be taken apart, washed with water, dried, and reassembled.

4. **Haldane's Hemoglobinometer.**—Haldane's hemoglobinometer is a very satisfactory instrument. Its only drawback—a minor one—is that illuminating gas is required in its use. Like the Sahli hemometer, it is a modification of the original Gower apparatus.

5. **Dare's Hemoglobinometer.**—Dare's hemoglobinometer gives good results, but is fragile and expensive.

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[14] Haden, R. L., *loc. cit.*

SULPHEMOGLOBINEMIA, METHEMOGLOBINEMIA

Sulphhemoglobin[15] occurs in the blood at times in association with an otherwise unaccountable chronic cyanosis, chronic diarrhea or constipation, and nervous symptoms, such as headache and vertigo.

The recognition of these abnormal pigments in the blood is described as follows by Clarke and Curts:[16] The blood is drawn from a vein, or, if this is not allowed, a few drops from the finger or ear will usually suffice. It is immediately diluted with twice its volume of distilled water, before clotting has taken place, and is thoroughly shaken. After the fibrin has separated, the solution is filtered several times through one filter paper, and the clear solution looked at through the spectroscope. The solution is then diluted drop by drop with water, until the red color (of the spectrum) stands out clearly. If there is a black absorption band in the red, either methemoglobin or sulphhemoglobin is present. If such a band persists after the addition of a drop of dilute ammonium sulphid, the pigment is sulphhemoglobin; if it disappears, it is methemoglobin.

In the blood the two bands of oxyhemoglobin are always visible. In addition to these, sulphhemoglobin presents a band in the red (near the orange) midway between *C* and *D*. With methemoglobin the band is again in the red, but nearer to C. (Compare with Fig. 9, p. 63.)

COLOR INDEX

The color index is the quotient obtained by dividing the percentage of hemoglobin by the percentage of red corpuscles, 5,000,000 cells per 1 c. mm. being considered as 100 per cent of corpuscles. Normally, the color index is about 1. When the index is less than 1, it indicates that the average corpuscle is poor in coloring matter, whereas with a high index the corpuscles are abnormally rich in hemoglobin.

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[15] Wallis, R. L. M. "On sulphaemoglobinaemia." *Quarterly Jour. Med.*, 1913, VII, 73. (Literature.)

[16] Clarke, T. W., and Curts, R. M. "Sulphhemoglobinemia, with a report of the first case in America." *Med. Record*, 1910, LXXVIII, 987.

VOLUME INDEX

The volume index of the blood was first studied by Capps.[17] He introduced the term to designate the quotient of the percentage volume of the erythrocytes divided by the percentage number of these cells.

The *volume index* is of interest primarily in the anemias. Capps finds that a more accurate estimate of the average size of the corpuscles can be obtained in anemia in this way than by measurement of the corpuscles, which is difficult and often misleading when poikilocytosis is present.

The cell volume is invariably increased in *pernicious anemia,* and the increase in volume index is usually more striking than the increase in color index. Capps studied thirty cases. In twenty-six cases of *chlorosis,* he found a lowered volume index as a rule, though the decrease in volume index was less than the lowering of the color index. The volume index was found to be of considerable prognostic significance in chlorosis; patients with normal or nearly normal volume index recover quickly, even though the hemoglobin is much deficient, whereas those with low volume index convalesce slowly.

In *secondary anemias,* as a rule, the volume index is only slightly lowered with anemia of moderate degree, but as the anemia becomes more severe, the index decreases. However, a small group of secondary anemias was studied, in which the color and volume indices were low in the early stages, as in chlorosis; later, as the red count fell, high color and volume indices were found, as in pernicious anemia.

In all anemias, Capps found that the loss in color was greater and more rapid than the loss in volume. During recovery, the volume is restored before the color reaches normal. He found that the cell volume suffers little change in cases of dropsy, cyanosis, hydremia following acute hemorrhage, and jaundice (with some exceptions).

Determinations of the volume index consume very little time, and could be included with advantage in routine blood examinations, particularly in anemias.

Method.—To determine the volume of the red corpuscles, the *hematokrit* is employed. The usual form of apparatus is a hand or electric centrifuge armed with a frame for carrying two capillary tubes. The tubes are graduated from 0 to 100. Of the various procedures which

[17] Capps, J. A. "A study of volume index. Observations upon the volume of erythrocytes in various disease conditions." *Jour. Med. Research,* 1903, X, 367.

have been proposed, Capps recommends the following: The capillary tube is completely filled with blood, the distal end of the tube smeared with vaselin, and placed in the carrier of the hematokrit. "Two conditions are essential to prevent coagulation, viz., scrupulous cleanliness of the tubes and speed in operation. The latter condition requires that the blood must be placed in the hematokrit within a few seconds of withdrawal. . . . It is desirable always to fill two tubes as a control of one's results. The machine should be operated for three minutes at a uniform speed of ten thousand revolutions a minute" (Capps). The tubes are now examined, and it is seen that the corpuscles have been thrown to the distal end, leaving the clear serum proximally. *With normal blood and a count of 5,000,000, the red corpuscles extend to the line marked 50, occupying one-half the capillary tube.* This is the normal, and represents 100 per cent volume. The erythrocytes are counted at the same time that the volume determination is made. The volume index $= \frac{\text{volume per cent}}{\text{number per cent}}$, 5,000,000 corpuscles being considered 100 per cent. *In normal blood the volume index is 1.* Owing to variations in the size of the erythrocytes in anemias, the percentage volume does not run parallel to the percentage number, as a rule.[18] The volume index, then, expresses the relative volume of the average red cell as compared with the normal.

In determining the volume of the red corpuscles, the leukocytes separate as a paler, grayish layer above the erythrocytes. Where their number is greatly increased, as in leukemia, determination of the volume of the red corpuscles is impossible.

The hematokrit furnishes a ready means of making macroscopic examination of the *blood serum.* Lipemia, cholemia, and hemoglobinemia may be revealed in this manner, if sufficiently marked, though hemoglobinemia may be an artefact from mechanical injury to the red corpuscles.

Cleaning the Hematokrit Tubes.—Blood should be blown out of the tubes as soon as the reading has been made. The tubes are cleaned by drawing water, alcohol, and ether through them successively. If they are not perfectly cleaned, use acetic acid first, then the other fluids in the order given.

[18] Capps (*loc. cit.*) reports extremely interesting observations on volume index compared with color index and with measurements of the erythrocytes in primary and secondary anemias.

MEASURING THE DIAMETER OF CELLS

The diameter or a dimension of microscopic objects is expressed in *micra* (designated by the Greek letter μ), one micron being the thousandth part of a millimeter (0.001 mm.). In making measurements an *ocular micrometer* is employed. This is a glass disc, on which fifty equal divisions are marked by parallel lines. The upper lens of the eye-piece is unscrewed, and the micrometer is inserted in the tube of the ocular.[19] The value of the divisions on the micrometer scale is now determined in the following manner: The magnification of the microscope is varied by three factors, namely, the objective, the ocular, and the tube length. The usual tube length employed is 160 mm. Using this, the value of the spaces on the micrometer is determined with the objective and ocular to be used by comparison with an object of known dimensions. The most convenient object for this purpose is the counting chamber. The ruled area is placed under the microscope, and the number of divisions of the micrometer scale, which fall between the opposite sides of one of the smallest squares, or, in the case of low magnifications, between the sides of a larger unit, is found. Knowing the dimensions of the ruled surface, it is a simple calculation to compute the value of a single division of the micrometer scale. The smallest squares are $\frac{1}{20}$ mm. on a side, or 50 micra.

As applied to the blood, measurements are usually made on stained films. At least one hundred cells should be measured, and, where much anisocytosis exists, two hundred cells should be the minimal number.

In the measurement of oval bodies, such as the eggs of many parasites, the two dimensions are readily obtained by rotating the ocular through ninety degrees.

VISCOSITY OF THE BLOOD AND OTHER FLUIDS

The viscosity of the blood is, in a general way, proportional to the red cell count. Thus, in cases of polycythemia, the viscosity is increased, whereas in the anemias it is decreased. This parallelism is not sufficiently close to permit one to predict accurately the number of erythrocytes, and viscosity determinations are, therefore, more a matter of scientific interest than of practical diagnostic aid.

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[19] In ordering an ocular micrometer, the name of the maker of the microscope should be given, as the micrometer of one make may not fit the ocular of another.

For clinical use, a number of instruments for determining viscosity have been described. That of Hess [20] has proved very satisfactory. It is compact and easily portable. The determinations may be made with a little practice in two or three minutes. The subject of viscosity is well discussed by Austrian.[21] The viscosity is compared with that of water.

Method of Hess.—The Hess viscosimeter (Fig. 80) consists of an opaque glass plate (*H*) on which two graduated tubes, *A* and *B*, are mounted. At one end these tubes communicate with a T-tube, *G*, which in turn is connected by rubber tubing with the rubber bulb *L*. At the other end the graduated tubes connect with capillaries *C* and *D*. The latter open into tubes *E* and *F*, which have the same diameter as the graduated tubes *A* and *B*. Capillary tube *C* and tube *E* are made in one piece, while tube *F* is held in apposition with tube *D* by means of

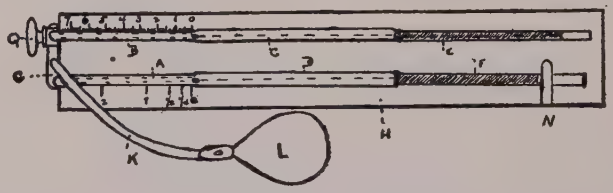

Fig. 80.—Hess' Viscosimeter.

the clip *N*. It is removable, and a number of similar tubes are supplied with the instrument. Through the valve *Q* it is possible to shut off the communication of the graduated tube *B* with the T-tube, and, therefore, with the rubber bulb as well. Between the rubber bulb and the rubber tubing a short piece of glass tubing is inserted; in it a hole is blown. This is opened or closed with the finger, and permits instant release of the negative pressure produced by the suction of the bulb. A thermometer is mounted on the glass plate.

Method.—With a pipette, which is furnished with the instrument, distilled water is placed at the opening of tube *E*. The valve *Q* is opened, and by suction from the bulb *L* the water is drawn into the tube *E*, until it reaches the capillary tube *C*. The pipette is then withdrawn, and the column of water is sucked further, until it reaches the

[20] Hess, W. "Ein neuer Apparat zur Bestimmung der Viskosität des Blutes." *München. med. Wchnschr.*, 1907, LIV, 1590.

[21] Austrian, C. R. "The viscosity of the blood in health and disease." *Johns Hopkins Hosp. Bull.*, 1911, XII, 9.

mark 0 on the scale of the graduated tube *A*. The valve *Q* is then closed, the pressure having been released by removing the finger from the opening. (It is unnecessary to refill the tubes *A, C, E,* with distilled water for each determination; the water may be allowed to remain in the tubes and used repeatedly.) The tube *F* is then touched to a fresh drop of the blood to be examined. The blood should enter at the pointed end of the tube. When the latter is about three-fourths full the tube is held so that the blood will run down to the funnel-shaped end of the tube, which is then placed in contact with the free end of the capillary tube *D,* and held in position by the clip *N*. By suction with the bulb the column of blood is then drawn to the line 0 on the scale of the graduated tube *B,* when the pressure is again released. The valve *Q* is now opened, and by suction through the bulb, the column of blood is drawn to the mark 1 on the scale. It is drawn exactly to the mark, when the pressure is removed by withdrawing the finger from the opening. The point on the scale to which the water has been drawn represents the degree of viscosity of the blood. The viscosity of the blood of normal adults is about 4.55 (Austrian). If the viscosity of the blood is very great, or if the blood coagulates rapidly, the column of blood is drawn to the mark ¼ or ½, and the result obtained is multiplied by 4 or 2 respectively. The error arising from making the observations at ordinary room temperatures is negligible (Austrian).

Cleaning the Viscosimeter.—As soon as the reading is made, positive pressure is exerted to expel the fluids from the graduated tubes. When the water reaches the zero line the valve *Q* is closed. The tube *F* is removed and the blood which escapes from the capillary tube *D* is caught on filter paper or cloth. A second tube, filled with concentrated ammonium hydrate, is placed in the clip, and ammonia is drawn through the tubes *A, D,* at least 2 cm. beyond the line 1 to which the blood extended. The ammonia is expelled and the tubes are refilled with fresh ammonia, which is allowed to remain in the tubes until the instrument is used again. The end of the capillary tube *D* is closed with a rubber cap. Immediately before using the apparatus the cap is removed and the ammonia expelled. If the pressure used to expel the ammonia is slight, only a trace remains, which is without appreciable effect on the result. It is essential that the tubes be perfectly clean. If the apparatus is unused for some time, difficulty may be experienced in forcing the ammonia out of the tubes. This is usually due to the formation of ammonium salts at the opening of the capillary tube; they may be

removed by solution in water. The valve should be lubricated with vaselin. The tubes *F*, after use, may be cleaned by aspirating water through them and then placing them in nitric acid for several hours. They are then dried by successive rinsings with water, alcohol, and ether. Erroneous results may be obtained if the tubes are dirty. The instrument should be tested from time to time with distilled water. If the result is not 1, the tubes are to be cleaned by drawing nitric acid into them. After an hour or so the acid is removed, the tubes rinsed twice with water, and then with ammonia.

THE SPECIFIC GRAVITY OF THE BLOOD

In clinical work, the method usually used for determination of the specific gravity of the blood is that of Hammerschlag. A mixture of chloroform (sp. gr. 1.485) and benzol (sp. gr. 0.88) is placed in a cylinder. The specific gravity of the mixture should approximate that of normal blood (1.050 to 1.062). A capillary tube is filled with blood, which is flowing freely from the puncture wound, and a drop is allowed to fall into the mixture. If the drop sinks, its specific gravity is greater than that of the mixture, and more chloroform is added; if the reverse holds good, benzol is added. After each addition of chloroform or benzol the contents of the cylinder are well stirred. When a mixture is finally obtained in which the drop of blood neither sinks nor rises, its specific gravity is determined with an aërometer. The result is approximately the specific gravity of the blood.

As Naegeli suggests, a series of mixtures of varying specific gravity is a great convenience.

The blood should not be permitted to drop into the chloroform-benzol mixture from a height, as it scatters. A bubble in the drop of blood may lead to serious error. Quick work is necessary to prevent the extraction of much water from the blood, and also to avoid evaporation of the mixture. After use the mixture may be filtered and kept in a brown glass bottle.

The *specific gravity of blood plasma or serum* may be determined by the method of Hammerschlag. Normally it lies between 1.029 and 1.032.

For the more accurate and time-consuming methods of determining specific gravity the reader is referred to works on hematology.

THE COAGULATION TIME OF THE BLOOD

The methods of determining the coagulation time are many and the results obtained with each are more or less divergent. Of the methods thus far proposed the most satisfactory, as well as the simplest, is that of Mills and Peterson.

Method of Mills and Peterson.[22]—The method depends on the fact that the blood will cease to flow back and forth in a capillary tube at the first sign of clotting. Capillary tubes of 0.6 to 0.8 mm. inside diameter are drawn from clean glass tubing and cut into lengths of about 1¼ to 1½ inches. Blood is obtained from a stab wound of the ear or finger, the first drop being discarded. The tube is touched to the second drop and allowed to flow in by capillarity, leaving about ¼ inch unfilled. Time is counted from the formation of the second drop over the wound. Slight pressure is permissible in getting the drop to form rapidly, provided it is applied at some distance from the wound. After filling, the tube should be gently inverted and observation made at the time when blood ceases to flow on inversion. Jarring or shaking should be avoided.

In order to get results under comparable conditions of temperature, the filled tube is placed in one of the creases of the palm of the hand and completely covered by closing the hand. This gives uniform temperature somewhat below that of the body. By opening the hand slightly for observation when inverting the tube, the end point may be noted.

The tube should be of sufficient diameter to allow the rather free flow of the blood when held upright just after filling. If the tube is too small, the attraction of the glass, a slight drying of the two ends of the blood column and an increase in viscosity all tend to impede the flow, with the result that the true end point cannot be distinguished.

With a clotting time of about 3 minutes, the tube should be inverted every 30 seconds; when beyond 5 or 6 minutes, it should be inverted every minute; and when less than 2 minutes, every 20 seconds.

The *normal* clotting time with this method is dependent upon the time of day when the determination is made—how long it has been since the last meal. It has been shown [23] that the normal clotting time

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[22] Mills, C. A., and Peterson, M. Personal communication. A method shortly to be published, for the use of which the author is indebted to his colleagues, Drs. Mills and Peterson.

[23] Mills, C. A., and Nakayama, J. T. Personal communication.

with no food in the gastro-intestinal tract is usually 3 to 3½ minutes. From 1 to 3 hours after each meal it is lowered to 1¼ to 2 minutes, rising again to 3 minutes usually before the next meal.

THE BLEEDING TIME

Bleeding Time (*Duke*).[24]—Clean the lobe of the ear with alcohol, and when it is dry, puncture it so that the blood flows freely, and note the time. As rapidly as a drop of blood forms, touch a piece of filter paper to it to absorb it. Take the time when a drop ceases to form. The normal bleeding time is 2 to 4 minutes. It is often noted that the bleeding time varies inversely with the platelet count, that is, when the platelet count is low, the bleeding time may be prolonged.[25]

THE RESISTANCE OF THE RED BLOOD CORPUSCLES

Numerous substances have been employed, against which the resistance of the red blood corpuscles has been measured. Solutions of sodium chlorid of varying strength, notably hypotonic solutions, have been most extensively used, and with them results of clinical importance have been obtained.

Method.[26]—Under aseptic precautions, 2 to 5 c.c. of blood are aspirated from an arm vein, and immediately placed in five to ten times the volume of 1 per cent sodium fluorid or 1.5 per cent sodium citrate in 0.85 per cent sodium chlorid to prevent clotting. As soon as the blood is discharged into the fluid, the flask is shaken well to insure thorough mixture. The blood-fluorid mixture is now centrifugalized at high speed to throw down the corpuscles. The supernatant fluid, containing the greater part of the blood plasma, is poured off. The plasma is then completely removed by washing the corpuscles three times in 0.85 per cent solution of sodium chlorid. After the last washing the supernatant fluid is pipetted off, leaving the erythrocytes at the bottom of the centrifuge tube.

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[24] From H. B. Weiss and R. Isaacs. *A Manual of Bedside and Laboratory Technic.* (Mimeographed). 1921.

[25] Gram, H. C. "On the platelet count and bleeding time in disease of the blood." *Arch. Int. Med.*, 1920, XXV, 325.

[26] Moss, W. L. "Paroxysmal hemoglobinuria; Blood studies in three cases," *Johns Hopkins Hosp. Bull.*, 1911, XXII, 238.

The hypotonic solutions of sodium chlorid diminish from 0.85 per cent by 0.03 per cent, the solutions being 0.82 per cent, 0.79 per cent, and so on, down to 0.25 per cent. They are quickly prepared by filling one 50-c.c. burette, graduated to $\frac{1}{10}$ c.c., with distilled water, and another with 1 per cent aqueous solution of sodium chlorid. Thus, to prepare 10 c.c. of 0.70 per cent sodium chlorid, take 7 c.c. of the 1 per cent salt solution and 3 c.c. of distilled water.

A series of small test tubes is appropriately marked and placed in a rack. To each there are added 3 c.c. of hypotonic salt solution and 0.03 c.c. (about one drop) of the red blood corpuscles. The salt solution and blood corpuscles are well mixed by shaking. (The tubes are, of course, perfectly clean and sterile, and are plugged with cotton.) After all have been filled, they are placed in the ice chest to prevent bacterial growth, and are allowed to remain until the red cells have settled to the bottom. For the lower dilutions this usually requires about two hours. The supernatant fluid is now examined for free hemoglobin, the presence of which shows that there has been laking of the corpuscles.

The tube of lowest dilution showing even a trace of hemoglobin in the fluid represents the so-called *minimal resistance.* That is, with this strength of salt solution the least resistant cells are "laked," their hemoglobin escaping from the cell membrane into the salt solution. The *maximal resistance* is found by noting the strength of salt solution in which *all* the red corpuscles are laked.

Normally, the minimal resistance, in terms of hypotonic salt solution, is about 0.47, the maximal resistance about 0.30.

Lowering of the resistance of the red cells has been found by Chauffard [27] to be one of the diagnostic characteristics of *congenital hemolytic jaundice.* The increased fragility of the corpuscles is demonstrable both with whole blood and with washed corpuscles. Laking may begin in this disease with as high concentration as 0.72 per cent sodium chlorid solution (minimal resistance), and may be complete in 0.45 per cent or higher (maximal resistance).[28] In *acquired hemolytic jaundice,* it

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[27] Chauffard, A. (*a*) "Pathogénie de l'ictère congénital de l'adulte." *Semaine méd.,* Paris, 1907, XXVII, 25; (*b*) "Les ictères hémolytiques." *Ibid.,* 1908, XXVIII, 49. *Also* Chauffard, A. and Troisier, J. "Deux cas d'ictère hémolytique." *Bull. et mém. Soc. méd. d. hôp. de Par.,* 1908, 3 s., XXV, 411.

[28] Thayer, W. S. and Morris, R. S. "Two cases of congenital haemolytic jaundice with splenomegaly. Observations on haemolytic jaundice." *Johns Hopkins Hosp., Bull.,* 1911, XXII, 85.

was shown by Widal and Abrami [29] that a similar increase in fragility exists, but they found that it may not be demonstrable if whole blood is used in the tests, whereas it becomes evident when washed corpuscles are employed.

TYPING BLOOD FOR TRANSFUSION

Blood Type Determination by Use of Citrated Sera [30] (*Macroscopic Method*).—There are four types of sera, as shown by Moss, and in selecting a donor for transfusion, it is necessary to secure an individual whose red blood corpuscles will not be agglutinated in the serum of the recipient.

For typing bloods, it is necessary to have known type II and type III sera,[31] obtained from blood that has been collected under sterile precautions and allowed to clot. The sera are citrated to a strength of 1.5 per cent (to prevent coagulation when the test is made); phenol in a strength of 0.25 per cent is added as a preservative.

With small caliber pipettes made by drawing out glass tubing, place a small drop of type II serum on one end of a glass slide, and a similar drop of type III serum on the other end of the same slide. With the smooth, rounded end of a glass stirring rod, a small drop of blood from the finger or ear of the person to be typed is transferred to, and thoroughly mixed with, each of the two drops of type sera on the slide, the rod being cleansed before each mixture. Now, tip the slide slowly from side to side over a white background and in a suitable light, and look for the appearance of agglutination, which is denoted by the appearance of a brick-dust, clumped precipitate in the drop. The agglutination should occur promptly, within a minute, as a rule.

Interpretation of Result.—If agglutination occurs in both drops, the individual tested belongs to group I (Moss' classification). If no agglu-

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[29] Widal, F. and Abrami, P. "Types divers d'ictères hémolytiques non-congenitaux avec anémie. Recherche de la résistance globulaire par le procédé des hématies déplasmatisées." *Tribune méd.*, Par., 1907, N. S., XXXIX, 711. *Also*, Widal, F., Abrami, P. and Brulé, M. "Types divers d'ictères hémolytiques. La recherche de la résistance globulaire par le procédé des hématies déplasmatisées." *Bull. et mém. Soc. méd. d. hôp. de Par.*, 1907, 3 s., XXIV, 1127; "Différentiation de plusieurs types d'ictères hémolytiques par le procédé des hématies déplasmatisées." *Presse méd.*, Par., 1907, XV, 641.

[30] From a *Manual of Bedside and Laboratory Technic*, prepared by Drs. H. B. Weiss and Raphael Isaacs, for use in the Medical Clinic of the Cincinnati General Hospital, 1921. (Mimeographed.)

[31] These sera may be purchased.

tination occurs in the blood mixed with type II serum, but is present in the mixture with type III serum, the individual belongs to group II. If, on the other hand, the corpuscles are agglutinated by type II serum but not by type III serum, the person belongs to group III. If no agglutination occurs in either drop, the individual belongs to group IV. When no agglutination occurs, the drops have a homogeneous, ground-glass appearance that is present even after the drop has dried.

The presence or absence of agglutination may be confirmed under the microscope with low power.

If agglutination is slow in occurring or takes place in only a very slight degree, it is advisable to test *directly* the agglutinative action of the recipient's serum on the donor's corpuscles, in the above described manner. While an individual never changes type, there is evidence to show that the agglutinin content of a given person's blood may vary in quantity from time to time.

It is necessary, of course, that both donor and recipient be typed, if typing is done. A direct testing of the donor's corpuscles in the recipient's serum makes this unnecessary.

Since the cells of group IV are not agglutinated by the serum of any other group, a member of group IV may serve as donor for his own or any other group (the "universal donor"). As group III cells are agglutinated by the sera of groups II and IV, a member of group III may act as donor for the small group I and his own group. Group II cells are agglutinated by the sera of groups III and IV, and therefore, a member of group I may act as donor only for his own group.

It has been found that group I comprises about 10 per cent of persons; group II about 40 per cent; group III about 7 per cent; and group IV approximately 43 per cent.

To summarize briefly:

Donor of group IV is suitable for his own and all other groups—the "universal" donor.

Donor of group III is suitable only for group I and his own group.

Donor of group II is suitable only for group I and his own group.

Donor of group I is suitable for his own group alone, as his corpuscles are agglutinated by sera of all the other groups.

Precaution.—In selecting a donor, an individual in good health should be chosen, and *syphilis* must be excluded by clinical and serological examinations.

THE EXAMINATION OF FRESH AND STAINED PREPARATIONS OF BLOOD

The first requisite in the preparation of fresh or dried films of blood is perfectly clean glassware.

The Cleaning of Cover Glasses and Slides.—Of the various methods used to clean glassware for blood work in the author's laboratory, the following has given the most satisfactory results, and is always dependable:

1. Immerse the covers (or slides) in concentrated sulphuric acid for about twenty-four hours.
2. Pour off the acid and wash in running water.
3. Drain off the water and cover the glassware with 95 per cent alcohol for an hour or longer.
4. Replace the alcohol with choloroform and dry the glassware as needed.

The covers should be dried with a perfectly clean cloth, free from lint. An old linen handkerchief which has been laundered many times is suitable. If the glassware is to be kept dry, it should be placed in a dust proof receptacle.

Ether may be substituted for chloroform, but is less satisfactory.

For blood work ¾-in. square cover glasses, No. 1, are the best. The 3 × 1-in. glass slides should be thin, with straight, even edges, if they are to be employed in making blood films. If cover glasses are used in making the films, the finish of the slide is less important.

EXAMINATION OF THE FRESH BLOOD

In the examination of the fresh blood, a procedure which is too generally neglected, the specimen is prepared in the following manner: The ear is pierced, but the puncture should not be so deep as to cause a very free flow of blood, since it is essential to be able to regulate the size of the drop accurately. Therefore, a small, superficial puncture is made, from which the blood will escape easily on *very gentle* pressure. (Pressure is to be avoided as far as possible, to prevent the dilution of the blood with tissue lymph. In grasping the ear the fingers should be at least ½ inch from the wound. It is better, of course, to obtain the blood without any pressure whatever.) A drop of blood about the size of a small pinhead is transferred to a cover slip, which is immediately placed upon a glass slide. The blood spreads out between the cover glass

and slide in a thin film. Microscopic examination should show the individual red corpuscles separated from one another in the central portion of the film, with the thicker parts at the periphery presenting rouleaux formation. If the cells are not separated, the drop of blood used was too large, provided the glassware was clean and the drop of blood fresh.

Failure to obtain satisfactory specimens is usually attributable to one of several causes. If the drop of blood is allowed to remain on the ear an appreciable length of time before it is used, clotting may have begun; this, of course, interferes with the proper spreading of the blood. Again, any dirt on the ear also has the same effect. Particles of dust or bits of lint on the glassware prevent even uniform spreading of the blood by elevating the cover glass from the slide. As dust frequently settles on the cover glasses or slides while preparing to secure the blood, it is a good plan to remove all such particles by blowing on the glass (avoid moisture from the breath on the glass), or by brushing the surfaces with a camel's hair brush. Any grease or dirt of any kind on the glass makes it impossible to obtain good specimens. It is advisable to handle the cover glasses with a pair of straight forceps, to avoid the grease, etc., of the fingers.

Sealing the Fresh Specimen.—If the specimen is to be kept for any length of time, it should be sealed to prevent drying. Vaselin is convenient for this purpose. A small quantity of it is taken up on the end of a match, which is then rapidly passed through a Bunsen flame. The edge of the cover glass is now lined with the melted vaselin, which hardens almost instantaneously, and effectually seals the specimen. Paraffin of low melting point may also be used. Specimens prepared in this way may be kept for a surprising length of time with little alteration in the red corpuscles.

THE PREPARATION OF DRY (PERMANENT) BLOOD SMEARS

1. *The Cover Glass-Forceps Method.*—In the writer's experience the best results are obtained by using two cover glasses. The covers are cleaned and dried as described on page 280. Any particles of dust are carefully removed from the covers just prior to making the smear. Forceps are used to avoid soiling the surfaces of the cover glasses with the fingers. With care, however, perfectly satisfactory films may be made with the fingers.

Two pairs of forceps are needed. One is a *cross-billed forceps,* which will hold a cover glass firmly. The spring should be strong and the blades perfectly parallel, so that the grip on the cover slip will be uniform. If the forceps are suitable it should be possible to lift them by grasping a cover glass caught between the blades of the forceps without changing the relative position of the cover glass. Forceps which cannot withstand this simple test usually prove to be useless. A pair of *straight forceps* is also required. They should be fairly stiff, with blades having plain, square ends. When holding a cover slip firmly *only the tips* of the blades should touch it.

To prepare blood films a clean cover glass is placed in the cross-billed forceps, the puncture wound is then wiped free of blood, and, when a drop of the proper size appears (about the size of a small pinhead with a normal count, larger with anemic blood), it is taken up on a second cover slip held in the straight forceps. This is immediately placed on the first cover glass. The blood spreads out between the two in a thin layer. Just *before* the drop will have stopped spreading between the covers, the overlapping edge of the second cover is grasped with the straight forceps, and the two are quickly pulled apart. It requires considerable practice to pull the covers apart in exactly parallel planes, which is necessary if the spreads are to be good. With good preparations microscopic examination will show the individual red cells well separated over one-half to two-thirds of the preparation. With a little experience good smears may be selected with the unaided eye. When inspected by transmitted light, the area in which the cells are properly separated resembles an extremely thin, gray veil; if the cells are grouped in little islands, the uniformity of the veil is lost. The thick parts of the smear are more dense and opaque.

The films, which are allowed to dry in the air, are then ready for fixing and staining. At times, when the humidity is very high, it may be necessary to fan the films to hasten the drying. (During the fly season films should be protected from the pests, as they may eat practically all the blood from a cover glass in a few seconds.)

The size of the drop of blood is a matter of great importance in making blood smears with the cover glass method, as has been indicated above. The correct size will depend largely on the number of red corpuscles in the blood. With very anemic patients, whose blood is thin and hydremic, a relatively large drop will be needed. The general tendency of beginners is to take a drop which is too large. If this mistake

is made, no part of the film is thinly spread, the erythrocytes being piled up so that study of the individual cells is impossible. If one waits until the blood has stopped spreading, it is often impossible to separate the covers, as they become sealed. Lint, dust, gritty particles, or grease on the cover glasses will make it impossible to secure satisfactory specimens.

2. *The Glass Slide Method.*—Many clinicians prefer glass slides to cover glasses in making blood films.[32] The method requires practically no practice, and is simpler than the cover glass-forceps method. The area of the blood film may be made much larger than that obtainable on a cover glass. The slides should be thin, and should have perfectly smooth, even edges and level surfaces. They must, as a matter of course, be perfectly clean. Any dust which may have settled on the slides should be removed before using them.

A drop of blood considerably larger than that required in the cover glass method is taken up on the end of one slide, which is then approximated to the surface of a second slide, placed on a table or other firm surface. The first slide is held at an angle of about 45 degrees to the second. The blood spreads out along the end of the first slide, which is now *pushed* rather rapidly along the surface of the second slide. The blood spreads out in a thin layer over the surface of the second slide. In making the spread, pressure on the slides is unnecessary.

Labeling the Blood Films.—With specimens made on glass slides, where the area of the blood film is large, a part of it may be employed for labeling the specimen. A very simple and practical method has been described by von Ezdorf.[33] The necessary data is written on the thick part of the film with a soft, black lead pencil. The label thus made is permanent, and is not affected by staining or washing the specimen. The black contrasts well with the usual pink color of the film.

Fixation of Blood Smears.—Various methods are available for fixing

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[32] In the experience of the author, more satisfactory specimens are obtained with the cover glass method. As a rule, the leukocytes are more evenly distributed over the specimen. The large smear, which is obtained with the slide, is usually no advantage, for it is seldom the case that a greater area is needed than is contained in a cover glass preparation. The great value of the slide method, aside from the fact that good smears may be obtained with it, lies in the fact that the technic is easily acquired, and fair specimens may often be obtained with slides which have been cleaned only with water. All laboratory workers should, therefore, be able to employ the method, though the cover glass method is preferred.

[33] Von Ezdorf, R. H. "The labeling of dried blood films." *Jour. A. M. A.*, 1910, LIV, 125.

the blood cells to the slide. When using Jenner's stain and most of the modifications of the Romanowsky stain (that is, all those staining mixtures which are dissolved in absolute methyl alcohol), previous fixation of the smears is unnecessary, as both fixation and staining are accomplished at one time. The following will be found useful:

1. *Heat Fixation.*—A triangular copper bar, first introduced into blood work by Ehrlich, is usually used for heat fixation. The bar is placed on a tripod with a Bunsen flame under the tip of the bar. In a short time, if protected from strong drafts, all parts of the bar acquire and maintain a fairly constant temperature. By dropping water from a pipette onto the bar, the point *farthest from the flame* is determined, at which the drop of water remains spheroidal and rolls off. The temperature at this point, the "spheroidal point" for water, is about 150° C. The point is marked, and the blood films, with the specimen side up, are then placed just inside this point, i. e., toward the flame from the spheroidal point, and allowed to remain 30 to 45 seconds. This usually suffices to fix the films well. In certain instances a longer or shorter time is required, the extremes falling between 5 and 120 seconds. By placing four specimens of blood (cover glass preparations) at the spheroidal point and removing them at the end of 30, 35, 40, and 45 seconds respectively, and staining all, the proper fixation time is quickly determined with the great majority of bloods. It is to be remembered that there is no one optimal fixation time applicable to all bloods. A separate determination must be made for each individual blood examined.

In place of the copper bar, an *oven* may be used. The specimens are placed in the oven, which is maintained at a temperature ranging between 110° and 120° C. for one to two hours, rarely longer. By removing and staining a specimen every fifteen minutes after the first hour, the correct fixation time is determined. This method of employing heat fixation is particularly convenient when a large number of specimens of the same blood are to be fixed.

Heat fixation is always used with Ehrlich's triacid stain. In using Pappenheim's methyl green-pyronin mixture, heat fixation is also to be preferred. It is less useful for other blood stains.

2. *Ethyl Alcohol.*—The specimens may be fixed by immersion in absolute alcohol one to five minutes, or in 96 per cent alcohol five to twenty minutes. They are then dried in the air or between blotting paper. Less expensive and about as satisfactory is the denatured alcohol of commerce.

This method of fixation is useful in connection with hematoxylin and eosin, methylene blue, etc.

3. *Methyl Alcohol.*—Absolute methyl alcohol, acting for one to five minutes, is an excellent fixative. (Methyl alcohol fixation is carried out as a part of the staining technic in employing Leishman's, Wilson's, and Jenner's stains, as will appear below.)

Methyl alcohol may be used in place of ethyl alcohol, and is usually employed with Giemsa's stain.

4. *Acetone.*—The specimens are placed in acetone five minutes, and are then dried in the air.

5. *Alcohol-Formalin.*—Futcher and Lazear have used 0.25 per cent formalin in 95 per cent alcohol. The solution must be prepared freshly, and is obtained by adding one drop of commercial formalin (40 per cent) to 10 c.c. of alcohol. The specimen is allowed to remain in this mixture one minute, and is then washed in water and blotted dry.

This method is the best for staining with carbol-thionin.

STAINING THE BLOOD

The stains and combinations of stain for blood work are numerous. Those described below are among the most serviceable, and enable one to make all routine examinations. Most of the stains are applied to dried, fixed films of blood. The staining of the fresh, unfixed blood, the so-called "vital" staining of the blood, forms an exception.

"Vital" Staining of the Blood

Various stains and methods have been proposed for vital staining of the blood. Practically any basic dye may be used for this purpose, but the stains which have been used most extensively are Unna's polychrome methylene blue (Grübler's), Pappenheim's methyl green-pyronin, and neutral red. Only the first is described in detail, since the picture obtained is rather more brilliant.

1. **Vaughan's Method.**[34]—A small puncture is made in the ear, and over the wound, from which the blood has been wiped, a minute drop of Unna's polychrome blue is placed by means of a clean glass rod. A small drop of blood is now pressed out of the wound, so that it flows

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[34] Vaughan, V. C., Jr. "On the appearance and significance of certain granules in the erythrocytes of man." *Jour. Med. Research,* 1903-4, X, 342.

directly into the stain. The procedure is now the same as in the preparation of a specimen of fresh blood (q. v.). The relative proportions of stain and blood are quickly learned by experiment. There should be more blood than stain in the mixture. After the specimen has spread out between cover glass and slide, it is ready for examination. It should be sealed, if the examination is to be a prolonged one.

On microscopic examination with the oil immersion objective, the majority of the red corpuscles appear quite like those in a preparation of fresh blood, except where the stain is concentrated; here the cells may show a diffuse purplish tint of varying intensity. Laking may occur in a certain number of the corpuscles. In some of the red cells granules stained bluish purple are seen. These basophilic substances have been designated "granulo-reticulo-filamentous" by Sabrazès.[35] There may be very few granules in a cell, or they may be extremely numerous. Often the granules appear to be attached to a delicate filament, which may form a part of a reticulum in the corpuscle. Not infrequently the granules are clustered at the center of the cell, suggesting by their position and number the remnants of a nucleus. In erythroblasts the nucleus takes a purple color, as do nuclear particles, when present. The chromatin of the blood platelets takes on a similar color, and may often be seen occupying a position at the periphery of a clear, unstained globule, as Vaughan observed. Leukocytic nuclei stain more or less intensely, depending largely on the concentration of the stain. In the protoplasm of the polynuclear cells, stained granules may be found. The ameboid leukocytes retain their activity for some time. The colorless cell membrane may be seen extending some distance beyond the granules in the pseudopods of the neutrophilic cells.

2. **Method of Widal, Abrami, and Brulé.**[36]—"Four to six drops of blood are allowed to fall into a test tube containing 10 drops of a basic coloring matter, which is quite isotonic, and contains in addition oxalate of potassium to prevent the coagulation of the blood.

Potassium oxalate, 20 per cent solution.....	2.0 c.c.	$\Delta = -0.60$
Unna's polychrome methylene blue.....	100 drops	

[35] Sabrazès, J., and Leuret, E. "Hématies granuleuses et polychromatophilie dans l'ictère des nouveaux-nès." *Gaz. hébd. d. Soc. Méd. de Bourdeaux*, 1908, XXIX, 123.

[36] Widal, F., Abrami, P., and Brulé, M. "Diversité de types des hématies granuleuses; procédés de coloration." *Compt. rend. Soc. de biol.*, Par., 1908, LXIV, 496.

"The fresh corpuscles are allowed to remain for 10 to 20 minutes in contact with the solution, after which the mixture is centrifugalized, the supernatant fluid is removed, and the corpuscles drawn up with a pipette and placed upon slides, upon which they are spread as an ordinary drop of blood; the covers are then dried and fixed by heat. Such preparations may be preserved indefinitely" (Thayer and Morris).

3. **The "Dry" Method of Vital Staining.**—The dry method of vital staining consists in spreading a thin film of stain on a glass slide, allowing it to dry in the air, protected from dust, and then placing a cover glass with a drop of blood on the dried stain, just as in making a preparation of the fresh blood. The blood spreads out between cover and slide, the stain dissolves in the plasma, and the result is much the same as with other methods. With this method there is less danger of laking the corpuscles.

Pappenheim's methyl green-pyronin mixture has been used extensively by the French, usually with the dry method.

Neutral red may be employed. A dilute solution of the dye is prepared in physiological salt solution, or a smaller quantity of saturated, aqueous solution of the stain may be used. It may be substituted for polychrome methylene blue in Vaughan's method, or may be used in the dry method.

Reticulated Red Cells

In *normal blood of adults* less than 1 per cent of the erythrocytes are reticulated on vital staining, while in *newborn infants* the figure is 7 per cent or less (Vaughan). Physiologically, there may be a striking increase in the percentage of reticulated corpuscles in those going to high altitudes (Barcroft).

In *anemias,* the presence of an *increased percentage of recticulated corpuscles* is good evidence of active regeneration of the blood. The percentage is often increased, as Vaughan showed, especially in *pernicious anemia.* The most striking increase is seen in *congenital or acquired hemolytic jaundice,* as first noted by Chauffard, and is one of the important differential diagnostic points, as an increase has not been observed in other types of jaundice.

The origin of the basophilic reticulation has not been definitely determined. The reticulation is absent in red cells normally nucleated, as in the blood of fowls, pigeons, etc. (Vaughan).

Reticulated corpuscles are those which, in permanent smears, show polychromatophilia.[37]

The Staining of Dried Blood Films

The stains which are used for the routine examination of blood are Ehrlich's triacid, Jenner's stain, and a Romanowsky stain. For special purposes, however, other stains are required at times.

1. **Methylene Blue.**—1. Fix the blood film in alcohol.
2. Stain with Löffler's methylene blue (p. 211) about 3 to 5 seconds.
3. Wash in water, blot dry, and mount in balsam.

The stain is useful as a nuclear stain. For the demonstration of basophilic granules and polychromatophilia, methylene blue is one of the most reliable stains.

Nuclei are stained dark blue. The leukocytic granules are unstained, excepting basophilic granules, which take a bluish purple color. The basophilic protoplasm so frequently encountered in lymphocytes is stained a paler blue than the nucleus, the shade varying greatly in different cells. The *erythrocytes* assume a pale greenish tint. Polychromatophilic red cells are light blue to very deep blue, depending on the degree of polychromatophilia. Basophilic granules in the red cells are stained dark blue, almost as dark as the nuclei. Nuclear particles in the red corpuscles take the same color as the nuclei of erythroblasts, i.e., a dark blue. *Blood platelets* are indistinct, appearing as dirty grayish blue masses.

2. **Eosin.**—Eosin may be used as a counterstain in ½ per cent aqueous solution. It is used after the methylene blue has been washed off the specimen. The stain is allowed to act a few seconds, the intensity of staining being controlled by microscopic examination of the film in water. Slower staining is secured by diluting the staining solution with water. Eosin adds little to the picture, except that it stains the eosinophilic granules, which now assume a brilliant pink or reddish pink hue. However, slight polychromasia may be somewhat less evident, though often more striking because of the contrast. The orthochromatic erythrocytes are stained pink. If the specimen has been overstained with eosin, the pink color will be apparent in the protoplasm of the lymphocytes and neutrophilic leukocytes.

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[37] Key, J. A. "Studies on erythrocytes, with special reference to reticulum, polychromatophilia and mitochondria." *Arch. Int. Med.*, 1921, XXVIII, 511. (Full discussion and literature.)

3. **Hematoxylin.**—Ehrlich's acid hematoxylin is prepared as follows:

Solution A:

Hematoxylin	2.0 gm.
Alcohol, absolute	60.0 c.c.

Dissolve.

Solution B:

Saturated solution of alum in equal parts of glycerin and distilled water	60.0 c.c.
Glacial acetic acid	3.0 c.c.

The two solutions, A and B, are mixed and allowed to "ripen" in an open bottle for a week. The bottle is then stoppered. The ripened stain has a reddish blue color. If the bottle is shaken or disturbed, the solution should be filtered before using.

Method.—a. Fix the blood films in alcohol. Heat fixation may also be used.

b. Stain in hematoxylin 2 to 10 minutes or longer. Control the intensity of staining by examining the specimen in water.

c. Wash in tap water. The washing may be completed in a few seconds, but the beauty of the nuclear staining is greatly enhanced by prolonged washing in tap water. If the specimen has been overstained with hematoxylin, it may be cautiously decolorized in acid alcohol (HCl, 1.0 c.c., 70 per cent alcohol, 100.0 c.c.), and again washed in water. It is better to avoid overstaining by controlling the staining carefully under the microscope.

d. Dry, mount in balsam.

Hematoxylin is one of the best nuclear stains. For studying the morphology of nuclei it is particularly useful.

Nuclei are stained a very dark blue, at times almost black. After prolonged washing, however, the blue is brighter—more brilliant. As with other nuclear dyes, the color intensity in a given nucleus depends, of course, on the amount and concentration of the chromatin. Mast-cell granules are stained dark blue, but may be lost after washing the specimen. Other leukocytic granules are unstained. Basophilic protoplasm is less intensely stained than with methylene blue. The *red blood corpuscles* are lightly stained, and are either gray or grayish blue. The more marked grades of polychromatophilia are revealed by the darker blue stain of the cells. Coarse basophilic granules in the erythrocytes are fairly well demonstrated as dark blue spots; the finer granules are

unstained or indistinct, as a rule. Nuclear particles take on a very intense, dark blue, like the pyknotic nuclei of normoblasts. *Blood platelets* are dirty blue and indistinct.

Eosin may again be employed as a counterstain. When the specimen is properly stained with eosin, no pink is seen in the protoplasm of the neutrophilic cells, while the eosinophilic granules stand out prominently. Hematoxylin and eosin are useful in cases where the relative number of eosinophilic cells is to be determined, as a differential count with this point alone in view may be made rapidly.

4. **Carbol thionin.**

Saturated solution of thionin in 50 per cent alcohol.	10.0 c.c.
Carbolic acid, 1 per cent.....	100.0 c.c.

a. Fix the blood films in alcohol formalin.

b. Stain with carbol thionin ¼ to 3 minutes.

c. Wash in water. If the specimen is overstained, the washing may be continued, or the specimen may be decolorized in 50 per cent alcohol.

d. Dry, and mount in balsam.

The stain is an excellent nuclear stain. All *nuclei* are stained dark blue. Leukocytic granules are not specifically stained with the exception of the granules of the mast-cells, which are purple. The *red blood corpuscles* are greenish gray. Basophilic granules are dark blue, polychromatophilic red cells varying shades of blue. Nuclei and nuclear particles are dark blue. The bodies of malarial parasites are purple, contrasting well with the red blood corpuscles. The nuclei of the parasites are unstained. *Blood platelets* are indistinct, and have a mauve color.

Preparations stained with carbol-thionin fade in the course of several months, as a rule.

Staining Mixtures of Two or More Stains

5. **Ehrlich's Triacid Stain.**—For the sharp differentiation of neutrophilic granules, the triacid stain of Ehrlich is unequaled. It should always be used in the study of these cells. The mixture contains three stains, two of which, orange G. and acid fuchsin, are acid, the third, methyl green 00, basic. The three basic radicals of the methyl green are satisfied by the acid dyes, hence the name, triacid. The formula [38]

[38] Morris, R. S. "The value of Ehrlich's triacid stain in blood work." *Jour. A. M. A.*, 1910, LV, 501.

given below, a slight modification of the usual one, has been found to yield uniformly good staining mixtures,[39] whereas formerly it has been more or less a matter of good fortune to obtain a satisfactory solution. Good staining mixtures may usually be had from Grübler. In preparing the mixture, saturated aqueous solutions of methyl green,[40] orange G., and acid fuchsin are made separately. They must be allowed to settle for at least a week before use, and should be replenished as needed, so that a constant supply of the stock solutions may be on hand. Grübler's stains are generally used.

The formula, as modified, is:

Saturated aqueous solution of orange G.....	13.0 c.c.
Saturated aqueous solution of acid fuchsin.....	7.0 c.c.
Distilled water	15.0 c.c.
Absolute alcohol	15.0 c.c.
Saturated aqueous solution of methyl green.....	17.5 c.c.
Absolute alcohol	10.0 c.c.
Glycerin	10.0 c.c.

The fluids are mixed with the same graduated cylinder, which should not be rinsed. The receiving flask should be shaken vigorously after the addition of each constituent, which is added in the order given in the formula. It is essential to add the methyl green, second portion of alcohol, and glycerin slowly, shaking well after each addition. The mixture is ready for use immediately, and does not deteriorate with age. After the mixture has stood a while, a small amount of precipitate may form. Care should be exercised that this is not disturbed when using the stain.[41]

Method of Staining.—a. Fix the blood spread by heat (p. 284).

b. Stain 5 to 10 minutes (overstaining is impossible).

c. Wash quickly in water, blot dry with filter paper, and mount in balsam.

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[39] Recently (1912) we have encountered the first failures in more than five years. After numerous experiments the cause of the trouble was found to lie in the acid fuchsin. Three different lots of the powdered stain in Grübler's original packages obtained through one firm resulted in poor staining mixtures, while acid fuchsin secured from another firm (also Grübler's make) gave very satisfactory results. With the poor acid fuchsin all cells were stained diffusely red. The nature of the defect in the acid fuchsin has not yet been determined.

[40] Methyl green is used in place of methyl green 00 of the original formula.

[41] For blood stains, bottles with droppers, the rubber nipples of which also serve as stoppers, are indispensable. Barnes' bottle is a very good one.

In a properly fixed specimen the neutrophilic granules stand out sharply. When this is the case the erythrocytes are usually, though not always, colored deep orange or buff; in an underfixed specimen they are stained red, while too prolonged fixation causes them to take a yellow color. The color of the red corpuscles, while a safe index of the fixation in most instances, fails at times. The final criterion by which a specimen is judged must be the staining of the neutrophilic granules.

In a good specimen (Pl. I) the *erythrocytes* have, then, a buff color usually. Polychromasia (see table, p. 303) is not demonstrated. Basophilic granules and Cabot's ring bodies are unstained. The pyknotic nuclei of normoblasts take a dark green color, the megaloblastic nuclei being less deeply stained. Often reddish areas are visible in the nuclei. Nuclear particles are stained green, but are much less striking than when stained with better nuclear stains, such as hematoxylin or a Romanowsky stain. Malarial and other parasites are not well stained. It is evident, therefore, that the triacid stain is very inferior for the study of pathological changes in the red blood corpuscles. Many of the abnormalities of the red cells are not shown at all, and none of them is as well demonstrated as with a Romanowsky stain or with Jenner's stain.

Blood platelets appear as ill-defined, indefinite, mauve-colored masses.

Leukocytic granules (see table, p. 303) are well differentiated, but the *nuclei* are poorly stained, assuming a rather pale green or bluish green color. All neutrophilic granules take a lilac color. There should be no blurring of them in a well-fixed specimen; the granules are sharply defined and distinct, though in the myelocytes the very fine granules stand out less prominently than in the polynuclear cells. Eosinophilic granules assume a brick-red or coppery color, while basophilic granules are unstained, appearing as vacuoles in the cytoplasm. The protoplasm of the lymphocytes is either colorless or a faint rose-pink. The same holds true for the large mononuclear and transitional cells; their nuclei, being poor in chromatin, usually stain very faintly, so that they are easily overlooked. Because of this difficulty with the non-granular leukocytes, Löffler's methylene blue has been used to stain the nuclei more intensely. It is applied to the blood film for a few seconds (3 to 5) after the staining with the triacid has been completed. The granular stain may be slightly impaired, but the nuclei are much more evident. The mast cell granules are now stained purple. In this connection it may be added that Pappenheim has prepared a triacid mixture, sub-

stituting methylene blue for methyl green, but it has not been widely adopted.

6. **The Romanowsky Stains.**—The Romanowsky stains are by far the best for the demonstration of pathological changes in the red corpuscles, and are, of course, indispensable in the study of such protozoa as the plasmodia of malaria, trypanosomes, etc. At the present time, they are employed in routine blood work, practically to the exclusion of other stains.

Romanowsky's original method of preparing the stain has undergone numerous modifications, with a view to simplification both of the preparation of the stain and of the staining technic. The essential dyes are eosin and methylene azure, the latter being obtained from methylene blue. Methods of preparing the stain have been described by a number of workers in this country, among whom may be mentioned Wright, Harris, Hastings, MacNeal, Wilson. Leishman's stain, which antedates all of those mentioned, is used extensively in England. The staining mixtures may be purchased; it is much more convenient and satisfactory, however, in private laboratories of physicians where only moderate amounts of stain are used, to buy tablets of the powdered stain. A tablet is dissolved in a stated quantity of absolute methyl alcohol (usually 10 c.c.), and the mixture is ready for use at once. In this way fresh stain may be had at frequent intervals, and there is less danger of deterioration of the mixture. Such tablets are prepared by Burroughs, Wellcome, & Co., and by the Coleman & Bell Co., Norwood, Ohio. The method of preparation of only one of the modifications of the Romanowsky stain, Wilson's, is given. The writer has employed it for several years with entire satisfaction.

7. **Wilson's Stain.**[42]—Prepare a 1 per cent aqueous solution of methylene blue,[43] which contains 0.5 per cent of sodium carbonate and at least 0.5 per cent of freshly precipitated silver oxid.[44] The solution

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[42] Wilson, T. M. "On the chemistry and staining properties of certain derivatives of the methylene blue group when combined with eosin." *Jour. Exp. Med.*, 1907, IX, 645.

[43] The cheaper grades of methylene blue may be used with satisfactory result.

[44] The silver oxid may be prepared by dissolving 2.0 gm. of silver nitrate in 15 c.c. of distilled water and adding to it 260 c.c. of calcium hydrate. Shake well, and set aside for the precipitate to settle. Decant the supernatant fluid, collect the precipitate on a filter, and wash with 20 to 25 c.c. of distilled water. Dry the precipitate at a temperature not exceeding 100° C., and place it in a brown bottle, tightly stoppered.

Legend for Plate I

(All drawings made with camera lucida; ×1200. Ehrlich's triacid stain.)

1, 2. Normal red corpusles.
3. Megaloblast.
4. Normoblast.
5. Lymphocyte.
6. Large mononuclear leukocyte.
7. "Transitional" leukocyte.
8. Polynuclear neutrophilic leukocyte.
9. Polynuclear eosinophilic leukocyte.
10. Mast cell or polynuclear basophilic leukocyte.
11. Neutrophilic myelocyte.
12. Eosinophilic myelocyte.
13. Mast myelocyte or basophilic myelocyte.
14. Myeloblast.

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R. S. M.

is boiled for twenty minutes; then remove one-third of it. After boiling another twenty minutes, remove one-half. Continue to boil the remaining portion twenty minutes. The three portions are now united and distilled water is added to the original volume, to compensate for the loss by evaporation. The mixture is allowed sufficient time for the precipitate to settle (about an hour). Now add an equal volume of 0.5 per cent aqueous solution of yellowish eosin (filtered) to the methylene blue solution in a large evaporating dish. Mix the solutions well, and allow the mixture to stand one hour. Filter thrice, using a hard filter paper, such as the Schleicher and Schüll filter, No. 575, and finally wash the precipitate which has collected on the filter paper with physiological salt solution. (The precipitate which adheres to the evaporating dish is discarded.) Dry the precipitate in the thermostat, and transfer it to a dark bottle, tightly stoppered. The staining solution is then prepared by dissolving 0.4 gm. of the powdered precipitate in 100 c.c. of absolute methyl alcohol (Kahlbaum's). The stain may be rubbed in a mortar with the alcohol to facilitate solution, or powder and alcohol are placed in a bottle, which is vigorously shaken a few minutes on several successive days. The staining solution should be preserved in a dark bottle with glass stopper. (Wilson advises the use of 0.3 gm. of the dry stain to 100 c.c. of denatured alcohol, but in our hands this has not given satisfaction.) It is best to make up small quantities of the stain at frequent intervals (3 to 4 months).

Method of Staining.—As the methyl alcohol in which the stain is dissolved is apt to run over the edge of the cover glass, it is advisable to use the usual wire staining forceps; when staining on glass slides, the stain may be confined to the area of the smear by drawing lines on the glass with a *blue* wax pencil—the red wax is usually loosened by the alcohol.

a. Cover the *unfixed* blood film with 5 to 6 drops of the stain for 1 minute. As the stain is dissolved in absolute methyl alcohol, the blood is fixed by this procedure. Precipitation of the stain through evaporation of the alcohol will be troublesome, if too little stain is used.

b. Add to the stain an equal number of drops of *distilled* water, and allow it to remain on the film 2 to 4 minutes. A metallic scum forms on the surface. (The exact proportion of stain and water should be determined for each new lot of stain. At times twice the quantity of water is necessary; occasionally, however, fewer drops of water than

of stain are required, especially with old mixtures, which have become slightly acid.)

c. Wash with *distilled* water, blot dry, and mount in neutral balsam. The specimen should be held level during the washing and the stream of water directed against the surface of the cover glass, so that the metallic scum and precipitate in the fluid will be *floated* off. *Avoid dumping the stain from the cover glass,* for the precipitate adheres to the corpuscles, and cannot be removed by washing in water. If there is precipitate in the specimen, it may be removed by immersing the preparation momentarily in absolute methyl alcohol or ethyl alcohol, but always at the risk of decolorizing the cells too much; it is particularly the basic stains, methylene blue and methylene azure, which are decolorized.

A properly stained blood film should have a pinkish gray or gray color when dry. If the color of the film is bright pink, the specimen is usually not satisfactory, or, rather, it is capable of being improved upon. The *erythrocytes* are stained very pale pink (Pl. II) or mauve or grayish pink. Polychromatophilia is denoted by varying admixtures of blue. In extreme grade a polychromatophilic red cell is dark blue, no trace of pink being discernible. (See table, p. 303.) Basophilic granules stain dark blue. Occasionally, particularly in the blood of pernicious anemia, fine granules are seen which are stained violet or purple. Nuclei are stained purple. Nuclear particles stain like the nuclei, while the ring bodies are usually violet or reddish purple. The differentiation of these abnormalities is more striking and brilliant with the Romanowsky stains than with any of the other blood stains. Cabot's ring bodies are usually demonstrable only with Romanowsky stains, that is, they are stained with methylene azure.

Blood platelets are well brought out with Romanowsky stains alone. The granular chromatin masses are stained reddish purple, the body of the platelet being unstained or exhibiting varying shades of blue.

All *leukocytic nuclei* (see table, p. 303) are beautifully stained, the color being a deep reddish purple. The morphology of the nucleus is well demonstrated. The leukocytic *granules,* on the other hand, stain poorly and very uncertainly, so that differential counting may be difficult or well-nigh impossible when pathological cells are present. The neutrophilic granules are stained lilac, but often only a few of the granules —sometimes none of them—take the stain. Eosinophilic granules are pink, but stain very uncertainly; at times there may be considerable

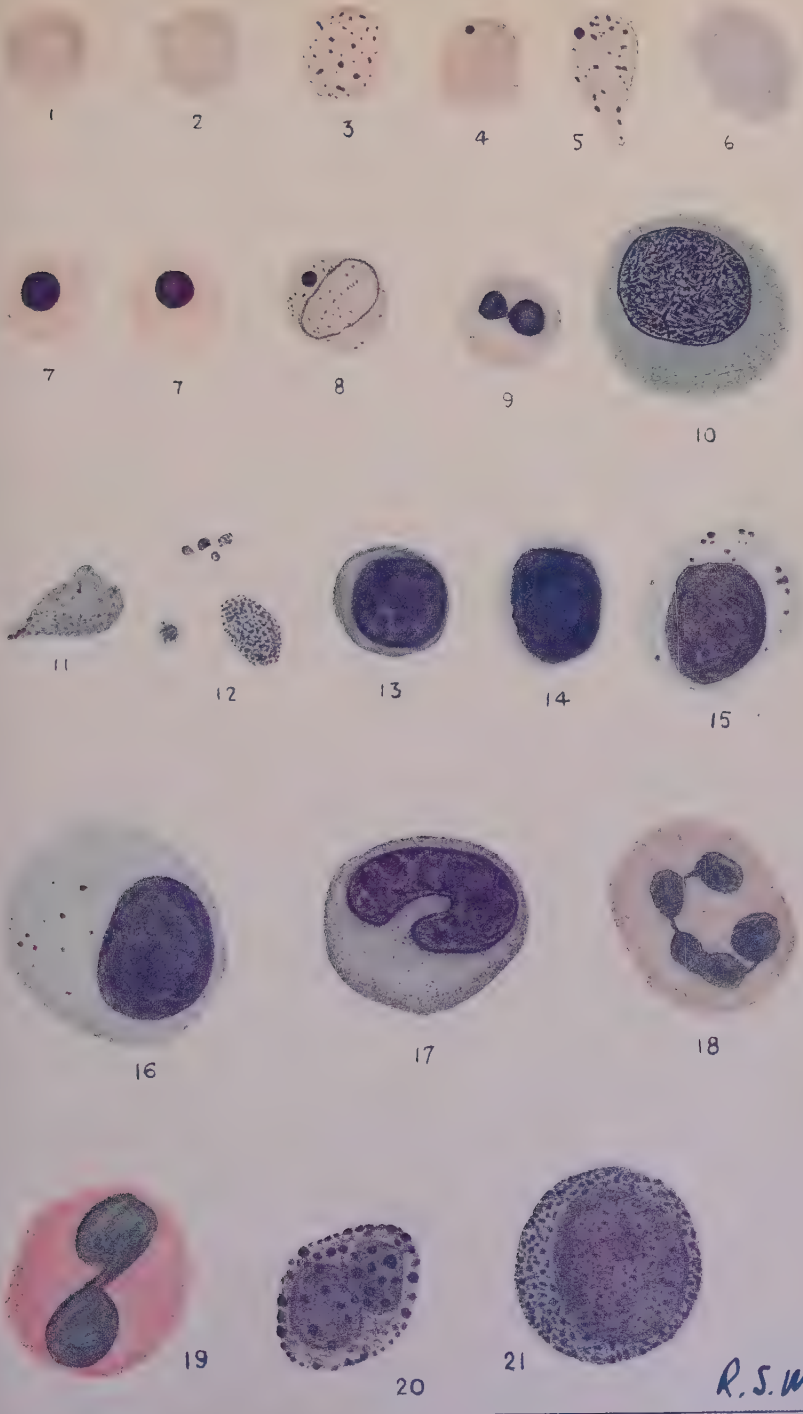
doubt as to their nature. Basophilic granules are colored purple. The granules of the myelocytes usually are purple, regardless of the variety of the cell, though eosinophilic myelocytes may show a shade of pink in the granules. The cytoplasm of lymphocytes is colorless or blue—at times a very dark blue. In about one-third of the lymphocytes of normal blood, purplish granules, varying much in size and number, are evident in the cytoplasm. These granules are demonstrable only with methylene azure, and are, therefore, designated *azurophile* granules. The large mononuclears and transitionals are, like the lymphocytes, more beautifully demonstrated with Romanowsky stains than by any other means. The cytoplasm of the large mononuclears is colorless or blue and generally nongranular, though it is now and then seen to contain azurophilic granules, which are for the most part very fine and dustlike. In the case of the transitional leukocytes the protoplasm is studded with very fine azurophilic granules practically without exception. When these granules are observed in a large mononuclear cell, the resemblance it bears to a myelocyte is close at first glance. It is seen, however, that, while similar in color, the myelocytic granules are rather coarser, and close inspection will usually reveal the granules over the nucleus in the myelocyte, a point which serves to differentiate them from the large mononuclears. Usually, too, the relations between nucleus and protoplasm are different in the two types of cell. (With the triacid stain and with Jenner's stain this difficulty never arises, since the large mononuclears are nongranular.)

Besides precipitated stain in the specimen, which may be avoided as indicated above (p. 296), the chief **difficulty in the application of the Romanowsky stains** arises in understaining with the basic components of the mixture. When this occurs, the erythrocytes are bright pink or red, the nuclei of the leukocytes blue instead of purple, and the chromatin of the platelets blue or unstained, while the chromatin of malarial or other parasites is entirely unstained. Such a condition may be due to one or more causes: (1) *Dilution* of the stain with too much water interferes with the nuclear staining. The requisite proportions of stain and water must be learned by experiment. If the nuclei are poorly stained, try less water. (2) Even a trace of *acid* in the water used for diluting or washing will weaken or remove the basic stains more or less completely. Staining in a room in which there are acid fumes is at times sufficient to ruin the specimen. (3) *Acidity of the staining mixture* itself may explain the failure of the nuclear stain. A minute

Legend for Plate II

(All drawings made with camera lucida; ×1200. Wilson's stain, modified Romanowsky.)

1. Normal red corpuscle.
2. Pale or anemic corpuscle.
3. Basophilic granules in erythrocyte.
4. Nuclear particle (Howell's body) in erythrocyte.
5. Erythrocyte containing nuclear particle and basophilic granules.
6. Polychromatophilic red cell containing a Cabot's ring body.
7. Normoblast.
8. Slightly polychromatophilic erythrocyte containing a nuclear particle, Cabot's ring body, and violet colored basophilic granules.
9. Normoblast showing an early stage of karyorrhexis.
10. Megaloblast, markedly polychromatophilic.
11. Poikilocyte, markedly polychromatophilic and exhibiting reddish basophilic granules.
12. Blood platelets.
13, 14. Small lymphocytes.
15. Large lymphocyte, exhibiting azurophilic granules.
16. Large mononuclear leukocyte with a few azurophilic granules in the cytoplasm.
17. "Transitional" leukocyte with fine azurophilic granules.
18. Polynuclear neutrophilic leukocyte.
19. Polynuclear eosinophilic leukocyte.
20. Mast cell or polynuclear basophilic leukocyte.
21. Neutrophilic myelocyte.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quantity of acid in the bottle (or on the cork) in which the stain is placed, or the formation of formic acid from the methyl alcohol, are possible sources of difficulty. Staining mixtures which contain acid may be made perfect, according to Peebles and Harlow,[45] by the addition of a few drops of absolute methyl alcohol in which a small quantity of potassium hydrate (by alcohol) has been dissolved. In case too much alcohol has been added, as shown by overstaining with the blue, it may be cautiously titrated back with absolute methyl alcohol containing a trace of glacial acetic acid. (4) *Prolonged washing* may spoil the result. It can readily be demonstrated by experiment that washing in water tends to remove the basic (blue) elements of the stain, thus rendering the eosin more conspicuous. With a good staining mixture and the right proportion of stain and water, it is only necessary to wash long enough to remove the excess of stain; the specimen is then blotted dry to prevent further decolorization.

Overstaining with the blue is seldom a source of difficulty, except in old smears. When it does occur, it may be corrected by one of the four procedures just described. With *very old blood films,* it may be impossible to prevent diffuse blue staining of the red corpuscles, though Brem's method (p. 162) or Giemsa's slow method may give good results.

8. **Leishman's Stain.**—This stain is most conveniently obtained in tablet form from Burroughs, Wellcome, & Co., or from the Coleman & Bell Co., Norwood, O., who also supply the powdered stain in bulk. A tablet is dissolved in a stated quantity of absolute methyl alcohol, and the mixture is then ready for use. It should be kept in a tightly stoppered bottle.

The staining technic is practically identical with that given for Wilson's stain. The nuclei have a little more of a reddish hue, but otherwise the picture is much the same (see table, p. 303). The advantages and limitations of the stain are those given above.

9. **Giemsa's Stain.**—The preparation of Giemsa's stain is difficult. The Coleman & Bell Co. supply a reliable solution. The staining mixture contains eosin, azure I, and azure II.

Method of Staining.—a. Fix the specimen in absolute methyl or ethyl alcohol.

b. Add one drop of the staining mixture to 1 c.c. of distilled water.

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[45] Peebles, A. R., and Harlow, W. P. "Clinical observations of blood stains." *Jour. A. M. A.,* 1909, LII, 768.

(This must be freshly prepared.) Stain 10 to 30 minutes with this dilution of the staining mixture.

c. Wash with distilled water, blot dry, and mount in balsam.

The appearance of the stained film is the usual Romanowsky picture (see p. 303). The nuclei, however, are a little redder than usual, and the neutrophilic granules are even less uniformly stained than with most other Romanowsky stains.

In case the *chromatin staining* is unsatisfactory, a very dilute solution of sodium carbonate may be substituted for distilled water in preparing the dilution of the stain.

Old Blood Films.—Blood films which have been kept for several months before staining them do not give good results with the usual Romanowsky procedures. The difficulty lies chiefly in the staining of the erythrocytes, which take a diffuse, slate-blue color. To avoid this to a great extent, the films may be stained with Leishman's or Wilson's stain, using Brem's technic (p. 162), or with Giemsa's stain. With the latter the dilution of the stain should be one drop to five or more cubic centimeters of water. The specimens are allowed to remain in this fluid 24 to 48 hours. They are then washed in water and mounted as usual. With this procedure it is often possible to demonstrate the chromatin of malarial parasites in specimens a year or more old.

10. **Jenner's Stain** [46] (the eosinate of methylene blue).—If one stain alone were to be selected for the general routine examination of the blood, Jenner's would possibly be the choice of some workers. It is a much better stain for nuclei and for pathologic alterations in the red corpuscles than Ehrlich's triacid, though inferior to the Romanowsky stains in these respects. On the other hand, it is much superior to the Romanowsky stains for the demonstration of the granules in leukocytes, though surpassed for this purpose by Ehrlich's triacid mixture.

Preparation of the Stain.—The tablets of Jenner's stain, which have been placed upon the market by Burroughs, Wellcome & Co., and by the Coleman & Bell Co., have done away with the necessity of making the stain, and have made it possible to have on hand fresh solutions of the staining mixture. The tablets are dissolved in a stated quantity of methyl alcohol; the solution is ready for use at once. Numerous modifications of Jenner's methods of preparing the stain have been

[46] Jenner, L. "A new preparation for rapidly fixing and staining blood." *Lancet*, 1899, I, 370.

described, without, however, simplification or improvement of the original procedures. Jenner's methods are as follows:

a. First Method.—Prepare a 1.2 per cent to 1.25 per cent solution of Grübler's water-soluble, yellowish eosin in distilled water, and also a 1 per cent aqueous solution of Grübler's medicinal methylene blue. Mix equal parts of the two solutions in an evaporating dish and, after stirring thoroughly, allow the mixture to stand for 24 hours. Filter through a hard filter paper (Schleicher and Schüll's No. 575), and dry the precipitate, which collects on the paper, either at room temperature or in the incubator at 37° C. The temperature may be as high as 55° C. without injuring the precipitate. The dried precipitate is removed from the filter paper, powdered in a mortar, shaken with distilled water, and the precipitate again collected on a filter paper. The washings should have a dirty purplish color. Finally, the precipitate is again dried and stored in brown glass bottles. For use dissolve 0.5 gm. of the powdered precipitate in 100 c.c. of absolute methyl alcohol, filter, and preserve in a tightly stoppered bottle. The solution keeps well.

b. Second Method.—Instead of using aqueous solutions, Jenner found that the eosin and methylene blue may be dissolved directly in absolute methyl alcohol. The staining mixture is prepared by adding 125 c.c. of a 0.5 per cent solution of Grübler's yellowish eosin in absolute methyl alcohol to 100 c.c. of a 0.5 per cent alcoholic solution of Grübler's medicinal methylene blue. The mixture is ready for use immediately.

Methods of Staining.[47]—The following is the technic originally recommended by Jenner:

a. Cover the *unfixed* blood film with the stain 1 to 3 minutes. To prevent evaporation and precipitation of the stain, the specimen is covered with a watch glass.

b. Wash quickly in distilled water, blot dry, and mount in balsam.

A *second method of staining*, which often gives good differentiation of the granules of the leukocytes and polychromatic nuclear staining, is as follows:

a. Cover the unfixed specimen with about 8 drops of stain for 2 to 3 minutes.

b. Add to the stain about 10 drops of distilled water, and allow the mixture to remain on the preparation 1 to 2 minutes or longer.

[47] In staining specimens on glass slides, where the area of the film is larger, more stain should be employed to prevent too rapid concentration of the stain through evaporation. The relative proportion of stain to water should be preserved.

c. Wash with distilled water (observing the precautions given on p. 296), blot dry, and mount in balsam.

With Jenner's stain the *red blood corpuscles* are terra cotta or pink. Polychromasia (see table, p. 303) causes the cell to assume a bluish tint. Basophilic granules in the red cells are dark blue, nuclei and nuclear particles are of the same color, though usually somewhat more deeply stained. Cabot's ring bodies are unstained or pale blue.

The *blood platelets* are poorly stained. They are mauve in color and indistinct morphologically. With the second method, however, they may be well differentiated, as with Romanowsky stains.

Leukocytic nuclei (see table, p. 303) are stained dark blue (purple with the second method). The neutrophilic *granules* are red, often with a violet tint. Eosinophilic granules are also red, but they are distinguishable from the former by their greater size and brilliance of staining. Mast cell granules are purple. The granular differentiation is less distinct in myelocytes than it is in polynuclear cells, as a rule, and is inferior to that obtained with Ehrlich's triacid stain.

The protoplasm of malarial parasites is stained light blue; chromatin is usually unstained.

Methyl Green-Pyronin Mixture of Pappenheim.[48]—Saturated aqueous solutions of methyl green and of pyronin are made. One part of the pyronin solution is added to three to four parts of methyl green. When sufficient pyronin as been added, the mixture begins to take on a bluish tint. It is often possible to obtain very good staining mixtures from Grübler.

Method of Staining.—a. Fix the blood films with heat.

b. Stain 5 to 10 minutes.

c. Wash quickly in distilled water, blot dry, and mount in balsam.

All nuclei are stained dark green or blue. Erythrocytes take a gray or slate color. Polychromatophilic cells are stained more or less intensely by the pyronin, and exhibit varying shades of red. Basophilic granules are stained a brilliant red, in marked contrast to the nuclei. Nuclear particles are stained dark green or blue,[49] like normoblastic nuclei, or red, like the basophilic granules.

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[48] Pappenheim, A. "Vergleichende Untersuchungen ueber die elementare Zusammensetzung des rothen Knochenmarkes einiger Säugethiere." *Virchow's Archiv*, 1899, CLVII, 19.

[49] Morris, R. S. "Nuclear particles in the erythrocytes." *Arch. Int. Med.*, 1909, III, 93. (The observations reported here were made on old films. Subsequent study of fresh material shows that nuclear particles, while not infrequently stained blue, are, nevertheless, often red.)

TABLE OF STAINING REACTIONS WITH EHRLICH'S TRIACID, ROMANOWSKY'S AND JENNER'S STAINS

Leukocytes	Ehrlich's Triacid Stain	Romanowsky Stains	Jenner's Stain
a. Lymphocytes.	Nucleus pale green, cytoplasm colorless or pinkish.	Nucleus reddish purple. Cytoplasm blue or colorless. Azurophilic granules in cytoplasm of *ca*. $\frac{1}{3}$ of lymphocytes.	Nucleus dark blue. Cytoplasm paler blue, at times darker than nucleus.
b. Large mononuclears.	Nucleus *very* faintly green. Cytoplasm unstained or pale pink.	Nucleus reddish purple. Cytoplasm blue or colorless. Fine azurophilic granules in cytoplasm, at times.	Nucleus blue. Cytoplasm paler blue.
c. Transitionals.	Same as *b*.	Same as *b*. Granules constantly present in cytoplasm.	Same as *b*.
d. Polynuclear neutrophiles.	Nucleus darker green or blue. Granules lilac.	Nucleus reddish purple. Granules lilac; often poorly stained or unstained.	Nucleus dark blue. Granules pink.
e. Eosinophiles.	Nucleus same as *d*. Granules copper colored.	Nucleus same as *d*. Granules pink, often poorly differentiated.	Nucleus same as *d*. Granules red.
f. Mast cells.	Nucleus as in *d*. Granules unstained, appear as vacuoles.	Nucleus same as *d*. Granules reddish purple.	Nucleus same as *d*. Granules purple.
g. Myelocytes.	Neutrophile, eosinophile and basophile granules well differentiated.	Usually all granules stain reddish purple.	Granules fairly well differentiated.
Platelets.	Poorly stained.	Well stained. Fine, purplish chromatin.	Poorly stained.
Red cells.			
a. Polychromasia.	Not demonstrable.	Varying shades of blue.	Varying shades of blue.
b. Basophilic granules.	Not stained.	Dark blue.	Dark blue.
c. Nuclear particles.	Pale green or blue.	Reddish purple.	Dark blue.
d. Ring bodies.	Not stained.	Violet or reddish purple.	Not stained or pale blue.
Malarial parasites.	Poorly stained. Chromatin unstained.	Chromatin red. Protoplasm light blue.	Chromatin usually unstained. Protoplasm light blue.

The protoplasm of lymphocytes is stained red. Leukocytic granules are not specifically stained.

The Iodin Reaction of the Leukocytes.—This reaction, discovered by Ehrlich, is demonstrated as follows: (1) The air-dried blood films are placed in a mixture composed of:

Iodin	1.0 gm.
Potassium iodid	3.0 gm.
Distilled water	100.0 c.c.
Gum arabic, q. s. (to give a syrupy consistence).	

(2) The air-dried specimen, instead of being placed in the mixture given above, may be put in a small vessel, in which a few crystals of iodin have been placed. The reaction appears in a few minutes. The specimen is mounted and examined in syrup made of levulose.

Permanent specimens cannot be made by either procedure.

The erythrocytes are stained diffusely brown.

The positive reaction consists in brown staining, of varying degree, of the protoplasm of the polynuclear neutrophiles. Occasionally the lymphocytes and mast cells are stained, rarely the large mononuclears and eosinophiles, while myelocytes never give the reaction (Zollikofer).

Differential Counting of the Leukocytes

For a differential count of the leukocytes, the first essential is a well spread and stained blood film. There is no one stain which is best for all purposes, but there is little question that one of the modifications of the Romanowsky stain (for example, Leishman's, Wright's, or Wilson's stain) gives the most satisfactory results in routine work. It is unsurpassed as a nuclear stain, it is the best for the demonstration of all the pathological changes in red cells and for the staining of platelets; and in the case of malaria, Leishman-Donovan bodies, and trypanosomes, it is the stain of choice. It is only in the differentiation of the granules in myelocytes that the Romanowsky stain is inferior to Ehrlich's triacid and Jenner's stains.

For special purposes, certain stains possess advantages. Thus, for the demonstration of neutrophilic granules, especially in myelocytes, Ehrlich's mixture is superior to other stains. Jenner's stain brings out the granules in neutrophilic myelocytes almost as well as Ehrlich's, and at the same time is better for staining eosinophilic and basophilic myelo-

cytes. In studying *eosinophilia,* when it is desired simply to follow the percentage of eosinophiles from day to day, hematoxylin or methylene blue combined with eosin may be used, always at the risk, however, of missing certain abnormal cells, if present. In *lymphoid leukemia,* where the lymphocytes may constitute 90 per cent or more of the leukocytes, it is advantageous to use a better nuclear stain than the triacid, and one which will differentiate between nucleus and protoplasm more effectively than Jenner's. The Romanowsky stains are the best for this purpose, for the staining is sharp and clear. Hematoxylin also gives excellent results, though the picture is less comprehensive, for all granules are unstained. The *large mononuclears* and *transitionals* are most satisfactorily demonstrated with the Romanowsky stains. The differentiation between nucleus and protoplasm is clear, the morphology of the nucleus is shown in great detail, and the azurophile granules are made evident.

For the differential count a *mechanical stage* is almost a necessity. The stained specimen is placed on the stage and examined with the $\frac{1}{12}$-in. oil immersion objective and an eye-piece (such as Leitz No. 3 or No. 4), which gives a high magnification. In making the count it is, of course, essential that the cells be counted only once. This is accomplished through the use of the mechanical stage, the specimen being moved up and down, with a lateral shifting of the field at the end of each "row." At least 200 cells should be counted.

Normal Leukocytes.—The leukocytes of the blood may be classified as follows:

1. *Lymphocytes* (Pl. I, 5; Pl. II, 13, 14, 15) are cells having a single round or oval nucleus—rarely a kidney-shaped nucleus—and a rather scanty rim of protoplasm. The protoplasm is nongranular, though about 30 per cent of the lymphocytes of normal blood possess azurophile granules, which are demonstrable after staining with methylene azure (i.e., Romanowsky stains), but not with other stains. These granules vary greatly in number and size. Often there are many granules, more frequently only a few, in a cell. The diameter of lymphocytes varies between 7 and 11 micra. They constitute 22 to 25 per cent of the leukocytes normally. In children under ten years, however, 40 to 60 per cent of the white cells are lymphocytes.

2. *Large mononuclear leukocytes* (Pl. I, 6; Pl. II, 16) resemble the lymphocytes, but are larger and have relatively more protoplasm. The nucleus is somewhat poorer in chromatin and, therefore, stains less intensely. The protoplasm may contain azurophile granules, which are

either very fine, like those of the transitionals, or rarely coarser, like the granules of the lymphocytes. The cells are actively ameboid and phagocytic—the *macrophages* of the blood. The diameter is 12 to 20 micra.

"Transitional" leukocytes (Pl. I, 7; Pl. II, 17) differ from the large mononuclears in the shape of the nucleus, which is horseshoe-shaped, lobulated, or deeply indented, and in the constant presence in the protoplasm of numerous fine, dustlike azurophile granules. With stains other than the Romanowsky, both cells present nongranular cytoplasm, though exceptionally a few fine, faintly stained granules are discernible after staining with Ehrlich's triacid stain. These granules, when evident, are usually stained a reddish or pinkish tint. (The diameter is the same as that of the large mononuclears.)

The large mononuclears and transitionals together form about 5 to 8 per cent of the leukocytes of normal blood.

Most workers at present consider the large mononuclears and transitionals to be derived from endothelium. The cells are often referred to as "endotheliocytes" or as "endothelial leukocytes." The transitionals are looked upon as older forms of the large mononuclears. The name "transitional," which has been in use since Ehrlich first introduced it many years ago, is a misnomer, and should be allowed to lapse.

In making the differential count, the large mononuclears and transitionals are grouped together.

3. *Polynuclear neutrophilic leukocytes* (Pl. I, 8; Pl. II, 18; Pl. IV, 1) are cells with polymorphous nuclei, in whose cytoplasm are numerous fine, neutrophilic granules. These cells are about 9 to 12 micra in diameter and constitute 65 to 70 per cent of the white cells under normal conditions. The cells are actively ameboid and phagocytic in the fresh blood and are designated *microphages,* in distinction to the macrophages or large mononuclears.

4. *Polynuclear eosinophilic cells* (Pl. I, 9; Pl. II, 19; Pl. IV, 2) are similar to the last group (4), except for the presence of coarse eosinophilic or acidophilic granules in the protoplasm. They resemble the neutrophiles in size and in the possession of ameboid activity. Normal blood contains about 2 to 4 per cent of these cells.

5. *Mast cells* (Pl. I, 10; Pl. II, 20; Pl. IV, 3) are *polynuclear basophilic leukocytes.* The nucleus is usually simply indented or lobulated. The protoplasm contains basophilic granules, which are somewhat variable in size, the majority being about as coarse as the eosinophilic

granules. The cells measure about 10 micra in diameter. Normally about 0.5 per cent of mast cells are found in the blood.

Pathological Leukocytes.—In addition to the foregoing cells, which go to make up the leukocytes of normal blood, there appear in disease immature cells, the precursors of the ripe leukocytes of normal blood.

6. *Neutrophilic myelocytes* (Pl. I, 11; Pl. II, 21; Pl. IV, 3) are the antecedents of the polynuclear neutrophilic cells. They differ from the latter in having a round or oval or slightly indented nucleus. The protoplasm contains neutrophilic granules, which are often finer than those of the polynuclear cells. In the older myelocytes the granules are abundant, while very young cells may contain only a few. The nucleus is poorer in chromatin than that of the mature polynuclear cell. The cells are subject to great variation in size. The majority lie between 12 and 20 micra in diameter, though larger and smaller cells are encountered now and then. In the nucleus one to four nucleoli may be visible, particularly after staining with methyl green-pyronin or with Romanowsky stains. The nucleus is usually eccentrically situated.

7. *Eosinophilic myelocytes* (Pl. I, 12; Pl. IV, 3) resemble neutrophilic myelocytes in every respect, aside from the difference in the granules. Frequently, the immature eosinophilic granules exhibit basophilic tendencies, in that they are stained with basic dyes. Thus, in a Romanowsky preparation, some or all of the granules may take a dark blue or purplish tint.

8. *Basophilic myelocytes* (*"mast" myelocytes*) (Pl. I, 13; Pl. IV, 3) are generally small and present basophilic granules in the protoplasm.

Myelocytes are found in the peripheral blood in largest numbers in myeloid leukemia. It is not unusual to find a few myelocytes (usually neutrophilic) in a marked neutrophilic leukocytosis; they are also encountered at times in connection with any disease which leads to overactivity of the marrow, as, for example, pernicious or other severe anemias, or malignant disease with bone marrow metastases.

9. *Metamyelocyte* is a term used to designate cells whose nuclei have passed beyond the kidney shape and already present more or less deep indentations. They are transition stages between the myelocyte and the polynuclear neutrophilic cells. They are not to be confused with the so-called transitional cells (which are, in reality, misbranded, as they represent transition forms to no type of cell, so far as is known, though it was originally supposed that they develop into the polynuclear neutrophiles, hence the name "transitional"). They are differentiated

from the transitionals by the abundance of neutrophilic granules in their cytoplasm, when stained with Ehrlich's triacid or Jenner's stain. With Romanowsky stains, on the other hand, the granules of the metamyelocyte and transitional may be identical in color, but those of the transitional are much finer.

10. *Promyelocytes* represent the earliest form of myelocyte, the cell with very few granules in its cytoplasm. The term is superfluous.

11. *Myeloblasts* (nongranular marrow cells, undifferentiated cells of the marrow, lymphoid cells of the marrow, etc.) (Pl. I, 14; Pl. IV, 3) are the parent cells of the myelocytes. They differ from the latter in the complete lack of cytoplasmic granules. The nucleus is similar to that of the myelocyte. The protoplasm is basophilic. The cells vary from the size of a lymphocyte to cells 20 micra in diameter.

Myeloblasts occur, often in large numbers, in myeloid leukemia. Isolated myeloblasts are often met with in other conditions in which myelocytes are found, but are much less distinctive than the latter morphologically in the stained smear. In the absence of myelocytes, their recognition, when only a few are present, is difficult, if at all possible, with the Romanowsky stains.

12. *Irritation forms* (Türk) are cells with round or oval nucleus, like that of the myelocyte, and rather abundant protoplasm, which is markedly basophilic and generally vacuolated. Türk's irritation forms may be encountered in connection with any condition which makes an usual demand on the marrow. They are frequently seen in leukocytoses, during convalescence from infections, in leukemias and anemias, et cetera. They are pathological myeloblasts (Naegeli).

13. *Pathological lymphocytes* (Pl. IV, 4, 5). In disease lymphocytes may depart considerably from the normal. The size is subject to much greater variation; the protoplasm is often greatly reduced in amount and at times is not demonstrable. The nucleus may be convoluted or indented, the so-called *Rieder cells.* They are not uncommon in lymphoid leukemia and in chloroma.

14. *Megakaryocytes,* the giant cells of the bone marrow, are very rare in the blood. The nucleus is greatly convoluted, and the cytoplasm, with Romanowsky stains, exhibits fine, dustlike granules. The cells are very large in the bone marrow, but only the smaller examples pass the capillaries and appear in the circulating blood.

The Normal and Pathological Red Blood Corpuscles

Non-nucleated red cells are designated *erythrocytes*, the nucleated forms *erythroblasts*.

Erythrocytes.—1. *Normocytes* (Pl. I, 1, 2; Pl. II, 1) are normal red blood corpuscles. In the fresh specimen they present the familiar form of biconcave discs, the center being paler, owing to the thinner layer of hemoglobin at this point. The normal cells stain with acid dyes (orthochromatic). The average diameter is 7.5 micra.

2. *Microcytes* (Pl. III, 1, 2), are abnormally small erythrocytes.

3. *Macrocytes* or *megalocytes* (Pl. III, 1), are abnormally large erythrocytes. They may be pale, swollen corpuscles or abnormally rich in hemoglobin. The diameter may amount to 25 micra. Abnormal variation in size of the corpuscles is designated *anisocytosis*. Ameboid movements may be observed in some of the cells.[50]

Macrocytes are conspicuous in many anemias, notably in pernicious anemia.

4. *Poikilocytes* (Pl. II, 11; Pl. III, 1, 2) are cells of irregular form. The shape may be practically anything. Poikilocytosis is not seen in normal blood, but is often a striking feature in severe anemias.

Erythroblasts.—*Nucleated forms* of the red blood corpuscle may be subdivided as follows:

1. *Normoblasts* (Pl. I, 4; Pl. II, 7, 7, 9; Pl. III, 1, 6) are nucleated red corpuscles, having the diameter of the average normocyte. The nucleus is round, at times eccentric, and usually very dense (pyknotic) and rich in chromatin. Younger forms present nuclei with a visible chromatin network.

2. *Microblasts* are abnormally small, nucleated red cells.

3. *Megaloblasts* (Pl. I, 3; Pl. II, 10; Pl. III, 1) are large erythroblasts, possessing a large oval or round nucleus. The diameter of the nucleus exceeds that of a normal red cell (Emerson). In the youngest forms the nucleus exhibits a beautiful chromatin network, with markedly basophilic protoplasm. More mature megaloblasts are more nearly orthochromatic and the nucleus is more homogeneous, the network being less conspicuous or lacking. Mitoses may be observed.[51] Ameboid activity may be seen in fresh specimens.[52]

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[50] Morris, R. S., and Thayer, W. S. "Amœboid movements in macrocytes and megaloblasts." *Arch. Int. Med.*, 1911, VIII, 581.

[51] Dock, G. "Mitosis in circulating blood." *Trans. Assoc. Amer. Phys.*, 1902.

[52] Thayer, W. S. "The amœboid activity of megaloblasts." *Arch. Int. Med.*, 1911, VII, 223. *See also* Morris, R. S. and Thayer, W. S. *Loc cit.*

4. *Intermediates* (Pl. III, 1) is a term used to designate all those nucleated red corpuscles which can be classified neither as normoblasts, megaloblasts, nor microblasts (Emerson).

Nucleated red cells in the peripheral blood are always pathological, except in *newborn infants,* where an occasional normoblast may be found. They may be encountered in all severe anemias (excepting aplastic anemias, where they are rare). Normoblasts, the oldest of the nucleated forms, are most commonly seen. Next in frequency come the intermediates. Megaloblasts are present in practically all cases of pernicious anemia, if searched for diligently; at times they are fairly numerous. They may be encountered, though rarely, in other anemias when very severe (in hookworm anemia, in chlorosis, for example). In irritative lesions of the bone marrow, such as malignant metastases, nucleated reds are frequently found in the blood. All forms of erythroblasts may be seen, too, in erythremia, though normoblasts are the rule.

The presence of nucleated red cells in the blood is evidence of increased marrow activity, and is, therefore, to be interpreted as a sign of regeneration.

Abnormalities in the Staining of the Red Corpuscles.—1. *Polychromasia* or *polychromatophilia* (Pl. II, 6, 8, 10, 11; Pl. III, 1, 3, 4) is a condition in which the red corpuscles stain with basic dyes. Normally the red cells possess an affinity for acid dyes, such as eosin. Polychromasia varies greatly in degree. With slight grades the color of the acid dye still predominates, though the tint of the basic dye is visible. When polychromasia is marked there is an intense staining of the cell with the basic dye alone. It occurs in both erythrocytes and erythroblasts.

Polychromatophilia [53] is seen in young red cells, and is, therefore, an evidence of regeneration. In fact, the rule is that *young cells in general have basophilic cytoplasm.* Encountered rarely in the peripheral blood of healthy adults, polychromatophilia is, nevertheless, constantly found in many of the red cells of the marrow, especially in embryos. Likewise, in the blood of normal embryos as well as in infants, polychromasia is normally found in a small percentage of the erythrocytes. In conditions in which there is an unusual demand on the bone marrow (anemias), the red cells enter the circulation prematurely; and it hap-

[53] Key, J. A. "Studies on erythrocytes, with special reference to reticulum, polychromatophilia, and mitochondria." *Arch. Int. Med.,* 1921, XXVIII, 511.

pens, therefore, that some of them stain more or less intensely with the basic dye (and therefore show varying tints of blue with the Romanowsky stains). Vital staining in such cases shows an increase in reticulated corpuscles.

2. *Basophilic granulation* or *stippling* (Pl. II, 3, 5; Pl. III, 1, 4, 5) is seen in non-nucleated red cells and in nucleated corpuscles, often with intact nuclei. As the name implies, the cell contains granules which are stained only with basic dyes. The granules are always quite numerous in the cell and vary considerably in size. As a rule, the smaller the granules the more numerous they are. With the variation in size there is also irregularity of form. Generally the larger granules are observed in orthochromatic cells, the smaller in polychromatophilic cells, as Askanazy has pointed out.

Basophilic granules in the red cells are also considered as evidence of active regeneration of the blood by the majority of hematologists. The old designation, "basophilic degeneration," should be dropped. The frequency with which basophilic granulation of the red cells is met with in lead poisoning has doubtless had much to do with the use of the term "degeneration." It has been shown, however, in experimental animals that basophilic granules appear in many of the erythrocytes after small doses of lead, whereas with large doses the cells showing the granules are few in number or are entirely absent. Furthermore, if a dilute solution of a lead salt is injected into an ear vein of a rabbit, circulation in the vein being prevented by a clamp, so that the lead and blood remain in contact in the vein, no basophilic granules form in the erythrocytes, though one would expect them, were they the result of degenerative changes in the cells caused by the lead. Basophilic granules may be found in the red cells in the normal bone marrow of embryos and infants, and at times in the marrow of normal adults. Whether the granules are derived from the nucleus, as many have supposed, remains to be demonstrated, but all the evidence points to the regenerative, rather than to the degenerative, nature of the granules.

3. *Fragmentation of the Nucleus.—Karyorrhexis* (Pl. II, 9), the breaking up of the nucleus within the cell, leads to characteristic appearances. The fragmented nucleus may resemble a rosette, or the nucleus may resolve itself into a number of round or oval, often irregular, masses, which are united or separate from one another.

4. *Nuclear Particles.*—Nuclear particles (Pl. II, 4, 5, 8; Pl. III, 1, 3) are derived from the nucleus of the red cell through atrophy of the

nucleus or by karyorrhexis; it is possible that there is another mode of formation, since nuclear particles may be found in megaloblasts with active, intact nuclei. They were first observed in the blood of the cat by Howell,[54] and are known as Howell's bodies or Howell's nuclear particles. They occur also in human blood. They are small, round, sharply defined bodies, usually situated eccentrically in the cell, and generally occur singly, though as many as nine have been observed in a non-nucleated red corpuscle. They resemble miniature pyknotic nuclei morphologically.

Nuclear particles are unquestionably evidence of the immaturity of the cells containing them, and are to be interpreted, therefore, as a sign of regeneration of the blood. They are found physiologically in the blood of embryos, and in the bone marrow of embryos and infants, rarely in normal adult marrow.[55]

The flooding of the blood with red cells containing nuclear particles, seen so frequently after splenectomy, indicates a connection with the spleen which is as yet unexplained.[56]

5. *Ring Bodies.*—Ring bodies (Pl. II, 6, 8) were first described by Cabot,[57] and are usually designated Cabot's ring bodies. The ring may remain round or may be twisted so as to form a figure eight, etc. The rings are best seen after staining with Romanowsky stains, which usually color them red or reddish violet, rarely blue. It is believed by Cabot, and by all who have since studied these bodies, that they represent the nuclear membrane. Ring bodies are generally looked upon as a sign of regeneration of the blood.

6. *"Red Basophilic Granulation with Romanowsky Stains"* (Pl. II, 8, 11).—Naegeli[58] in particular has called attention to the existence of a granulation seen in specimens stained with Giemsa's stain; it is

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[54] Howell,W. H. "The life-history of the formed elements of the blood, especially the red corpuscles." *Jour. Morphol.*, 1890, IV, 57.

[55] Morris, R. S. (*a*) "Note on the occurrence of Howell's nuclear particles in experimental anaemia of the rabbit and in human blood." *Johns Hopkins Hosp. Bull.*, 1907, XVIII, 198. (*b*) "Nuclear particles in the erythrocytes." *Arch. Int. Med.*, 1909, III, 93.

[56] Morris, R. S. "The occurrence of nuclear particles in the erythrocytes following splenectomy. *Arch. Int. Med.*, 1915, XV, 514.

[57] Cabot, R. C. "Ring bodies (nuclear remnants?) in anaemic blood." *Jour. Med. Research*, 1903, IX, 15.

[58] Naegeli, O. *Blutkrankheiten und Blutdiagnostik.* Leipzig, 1912, 2d ed., p. 153.

demonstrable with all Romanowsky stains. The granules differ from the usual basophilic granules, in that they are stained red or violet instead of blue. The granules were observed by Cabot in cells which also contained ring bodies. Naegeli believes that the granules originate from the nucleus, probably from the nuclear membrane, since ring bodies may be made of a series of dots similarly stained.

(1) to (6) inclusive are generally looked upon as evidences of *increased regenerative activity of the bone marrow.* In other words, cells showing the changes described are *young* cells. They may be found often in the marrow of normal embryos, where there can scarcely be a likelihood of degenerative changes.

7. *Schüffner's Granules.*—Schüffner's granules (Pl. V. [A] cell no. 5) are found only in certain cases of malaria. They are seen in the infected corpuscles (see below, p. 331).

EVIDENCES OF REGENERATION OF THE BLOOD (ERYTHROPOIESIS)

The evidences of regeneration (increased marrow activity) are:

1. An increased percentage of *reticulated red cells* (p. 287).
2. *Polychromatophilia* (p. 310).
3. *Basophilic granulation* of the red cells (p. 311).
4. *Nuclear particles* in the red cells (p. 311).
5. *Ring bodies* in the red cells (p. 312).
6. *Nucleated red cells* (p. 309).
7. *Eosinophilia* (that is, in severe anemia, with no discoverable cause for eosinophilia, especially in pernicious anemia, an increase in the percentage of eosinophiles often precedes a rise in the red cell count).
8. *Myelocytes* (that is, in severe anemias, in erythremia).

CHANGES IN THE BLOOD IN DISEASE

There are comparatively few diseases in which one can make the diagnosis from the blood alone. The leukemias and malaria may be recognized from blood examination, and the same is often true of pernicious anemia. Yet, it must be borne in mind that even the blood picture of a leukemia is not conclusive, as it has been observed to occur, for example, in miliary tuberculosis. All of the blood changes associated with primary pernicous anemia have been found in certain cases of

infection with the broad tape-worm, while chlorotic changes are not uncommonly met with, as, for example, in tuberculosis. It can safely be said that the blood offers the possibility of a *final* diagnosis only in those diseases, in which the causative agent is discoverable in the fresh or stained smear; malaria, trypanosomiasis, leishmaniasis, relapsing fevers, and filariasis are examples.

The majority of the alterations of the blood picture in disease are purely *secondary phenomena,* usually not in themselves pathognomonic, though often of great assistance, frequently indispensable, in arriving at the correct diagnosis of the patient's condition. *Given a set of symptoms and physical findings, a blood examination may practically clinch the diagnosis.* As examples, one may cite the eosinophilia occurring in trichinosis (Pl. IV, 2), the basophilic granulation in chronic lead poisoning (Pl. III, 4).

The following descriptions are intended to assist the student in visualizing the alterations most frequently found in certain diseases with pronounced changes in the blood. It is to be remembered that, in a given case, one finds rather infrequently *all* of the alterations forming the picture, which, after all, is a composite arrived at from the study of groups of patients.

PRIMARY ANEMIAS

1. **Pernicious Anemia** (Addison's anemia). **(Pl. III, Fig. 1.)**

Red Cells.

a. Reduction in the number of red corpuscles.

b. Reduction in the percentage of haemoglobin, not so great proportionately as the decrease in number of cells, resulting in a high color index, and an increase of haemoglobin in the individual red cells.

c. Increase in the average size of the red corpuscles (*macrocytosis*). Volume index increased.

d. Marked variation in size of the red cells (anisocytosis).

e. Irregularities in form of the corpuscles (poikilocytosis).

f. Evidences of regeneration: polychromatophilia, basophilic granulation, nuclear particles, Cabot's rings, nucleated red cells.

g. Megaloblasts. These cells are found in practically every case, and, while not diagnostic, are an essential element in the blood picture, often lacking at the first examination, but found if

the search for them is continued. These, with macrocytosis, constitute the most important elements in the blood picture.

White Cells.

Leukopenia, with relative lymphocytosis. The polynuclear neutrophilic cells in some instances are large and show an unusual number of lobulations in the nucleus, 6 to 7. An occasional neutrophilic myelocyte is not uncommon. There may be an eosinophilia, especially during periods of active regeneration. A *blood crisis* (that is, 50 or more nucleated reds per 1,000 leukocytes) may cause an *apparent* increase in the leukocyte count.

Blood Platelets.

The number of platelets is usually much diminished.

Aplastic Anemia.[59]—Probably due to aplasia of the bone marrow, either primary, or secondary, as in leukemia, severe infections at times, etc. In the primary form, there is failure in production of red cells, leukocytes, and platelets.

Red cells show gradual reduction in number, with comparatively little change in shape or form, and with little or no evidences of regeneration, such as nucleated reds, stippling, etc.

Hemoglobin is reduced proportionately to red cells, the color index being 1 or less.

Leukocytes.

There is leukopenia, due to decreased production of the marrow cells of the blood, that is, neutrophiles, eosinophiles, mast cells.

Platelets are markedly reduced in number.

The *bleeding time* is prolonged, likewise the coagulation time.

Chlorosis.—(Pl. III, Fig. 2).

Red cells normal in number or decreased; pale; average size decreased, with moderate anisocytosis and poikilocytosis in advanced cases.

Volume index decreased.

Few, if any, evidences of regeneration—an occasional cell showing polychromatophilia and rarely a normoblast.

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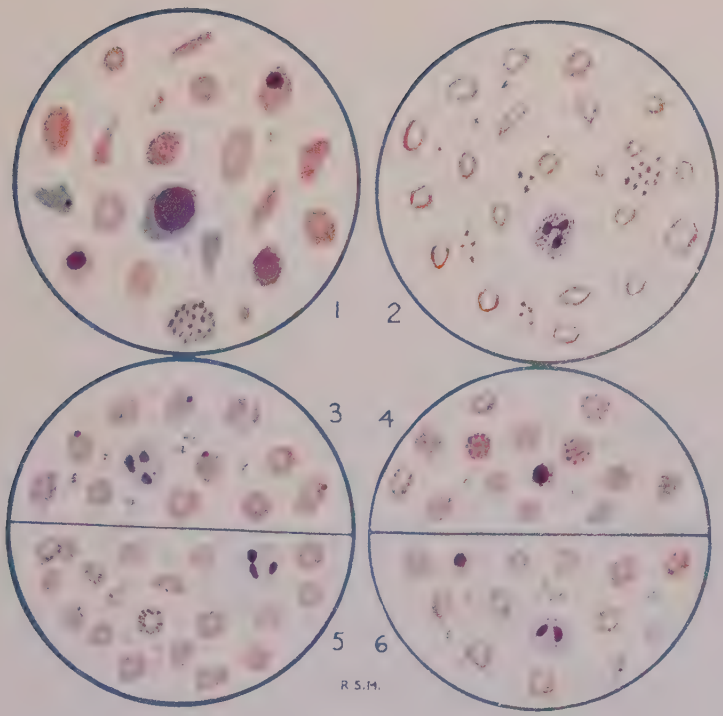
[59] Larrabee, R. C. "Aplastic anaemia and related conditions." *Jour. A. M. A.*, 1921, LXXVI, 1632.

Legend for Plate III

(Wright's stain; ×800.)

1. Primary pernicious anemia (combined field).
2. Chlorosis.
3. The blood following splenectomy.
4. The blood in lead poisoning (combined field).
5. Chronic post-hemorrhagic anemia.
6. Advanced secondary anemia in carcinoma of the stomach.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Haemoglobin much *decreased,* quite *out of proportion to the decrease in number of red corpuscles,* resulting in a *low color index,* and less than the normal amount of hemoglobin in the individual red cells; pallor of the red cells.

White cells normal in number, occasionally a slight increase. Differential count normal, or a moderate increase in polynuclear neutrophiles.

Platelets. Usually increased.

SECONDARY ANEMIAS

The so-called secondary anemias (Pl. III, Figs, 3, 4, 5, 6), as in malignant disease, lead poisoning (Pl. III, 4), hook-worm infection, bothriocephalus infection, etc., often conform more or less to the type of one of the two primary anemias. If the color index is low, the red cells pale, the anemia is spoken of as a "chlorotic" type of anemia, whereas, with cells rich in hemoglobin, with many large red cells, and color index of one or greater, the anemia is of the "hemolytic" type. The blood picture following splenectomy is characterized by many red cells containing nuclear particles (Pl. III, 3).

Among the commoner causes of secondary anemia may be mentioned:

1. **Hemorrhage** (including hemorrhagic diseases).
   a. Acute—large hemorrhage.
   Anemia—reduction in reds and hemoglobin.
   Leukocytosis, neutrophilic, moderate.
   Platelets increased.
   b. Chronic—repeated small hemorrhages.
   Anemia of chlorotic type (Pl. III, 6).
   Leukocytes normal.
   Platelets normal. (*Purpuras*—platelets decreased, bleeding time increased).
2. **Streptococcus septicemias.**
   Chlorotic anemia.
   Neutrophilic leukocytosis; white count at times normal or decreased.
   Platelets normal or decreased. Bleeding time often increased.
3. **Acute rheumatic fever.**
   Chlorotic anemia.
   Neutrophilic leukocytosis.

4. **Other acute infections,** especially when active several weeks.
   Usually chlorotic anemia.

5. **Malaria.**
   Anemia of chlorotic type.
   Leukopenia, with increase of large mononuclears, during apyrexial periods. *Pigmented leukocytes* (Pl. V).
   Neutrophilic leukocytosis during pyrexial periods (Thomson) (inconstant).

6. **Tuberculosis.**
   Chlorotic anemia; red count may be normal.
   Leukocytes normal, lymphocytosis.
   After septic infection (secondary), neutrophiles increased.

7. **Syphilis.**
   Chlorotic anemia, occasionally of the pernicious type; red count may be normal.
   Leukocytes generally normal, lymphocytosis during secondary stage. Large mononuclear increase during tertiary stage at times.

8. **Intoxications.**
   a. Lead. Chlorotic anemia; basophilic granulations of red cells. Red count may be normal (Pl. III, 4). Normoblasts common. Nuclear particles.
   Leukocytes normal; at times neutrophilic leukocytosis; leukopenia rare. (In 25 cases in the Cincinnati General Hospital, 3,500 to 14,400 w.b.c. Below 5,000 only 4 cases) (Hachen).
   b. Potassium chlorate.
   Marked anemia.
   Methemoglobin (p. 268).
   Neutrophilic leukocytosis.
   c. Nitrobenzol poisoning.
   Anemia, macrocytic; normoblasts, megaloblasts. C. I. + 1.
   Methemoglobin.
   Neutrophilic leukocytosis or normal.
   d. Arsin.
   Anemia.
   Hemoglobinemia (Joachim).
   Neutrophilic leukocytosis.

e. Benzol.

Anemia, severe.

Leukopenia.

Relative lymphocytosis.

f. Copper sulphate poisoning.

Marked anemia; C. I. — 1. Normoblasts and megaloblasts (Pollak).

Neutrophilic leukocytosis (myelocytes reported).

9. **Chronic nephritis,** especially acute and chronic parenchymatous.

Chlorotic anemia.

Normal leukocytes.

10. **Hemolytic jaundice,** congenital and acquired.

Well marked secondary anemia.

C. I. ± 1. *After splenectomy,* nuclear particles numerous (Pl. III, 3).

Resistance of corpuscles to hypotonic salt solution (p. 276) decreased.

Basophilic reticulation of reds on "vital" staining (p. 285), up to 20 per cent or more of the erythrocytes.

Leukocytes normal. Lymphocytosis inconstant.

11. **Banti's disease.**

Anemia, chlorotic. C. I. at times 1.

Leukocytes normal, or leukopenia with relative lymphocytosis.

12. **Animal parasitic infections.**

a. Hookworm.

Chlorotic type of anemia; very light infections, normal count, but hemoglobin is somewhat reduced.

Leukocytes normal.

Eosinophilia, moderate (inconstant).

b. Strongyloides stercoralis.

Reds normal, or chlorotic anemia.

Leukocytes normal, or less often increased.

Eosinophilia (to 45 per cent) (inconstant).

c. Dibothriocephalus latus.

Anemia of the pernicious type, the blood picture duplicating that of primary pernicious anemia. C. I. usually + 1.

Leukocytes normal or decreased. Eosinophilia inconstant; when present, only moderate.

d. Schistosomiasis.
Chlorotic anemia.
Leukocytes normal or increased.
Eosinophilia, 10 to 53 per cent (Naegeli).

e. Other parasites. Blood changes less constant. May be eosinophilia.

13. **Carcinoma.**
No anemia, or chlorotic anemia, which may be severe (Pl. III, 5).
Rarely the anemia resembles pernicious anemia.
Leukocytes normal or moderately increased. (Rarely, marked neutrophilic leukocytosis. With bone marrow metastases, nucleated reds and myelocytes common.)

14. **Other chronic wasting diseases.**
Chlorotic anemia.

POLYCYTHEMIAS

Polycythemia.—An increase in the number of erythrocytes, amounting to a total count as high as 15,900,000 per c.mm. (personal observation), may be encountered. The increase, as in the case of the leukocytes, is either symptomatic (*erythrocytosis*) or is due to an essential disease of the blood-forming organs (*erythremia*). There is a coincident increase in hemoglobin in nearly all cases, though it is relatively less than the increase in red count.

Erythrocytosis.—This may be found in the following conditions:

1. In those residing at *high altitudes* (six to eight millions).
2. In *circulatory insufficiency* and in *pulmonary emphysema* (six to nine and three-tenths millions).
3. In *congenital pulmonary stenosis* (six to eleven millions).
4. In *chronic infections* (tuberculosis [of the spleen] and syphilis [of the pulmonary arteries, Ayerza's disease]).
5. In *carbon monoxid poisoning* (eleven and two-tenths millions, but hemoglobin only 90 per cent, (Naegeli).
6. In *phosphorus poisoning.*
7. In *severe enteritis* (cholera), (concentration of blood).

Erythremia.—Probably a primary disease of the blood-forming tissues.

Red cell count increased to 7,000,000 to 15,900,000.[60] The cells may show pallor. Polychromatophilia is common, and normoblasts are of frequent occurrence. Megaloblasts may be found (Türk).

Hemoglobin is increased, but the increase is relatively less than the increase in red cell count. Values up to 200 per cent have been reported.

White cells are normal in number or are increased to 12,000 to 20,000, rarely to 50,000 or even higher, with an increase in percentage of polynuclear neutrophiles. Myelocytes are usually present, even when the total leukocyte count is normal, and may amount to 4 per cent. Eosinophiles and mast cells are frequently considerably increased (that is, absolute increase, at times also relative increase).

Platelets.—Abnormalities have not been noted.

The *viscosity* and *specific gravity* of the blood are increased.

THE LEUKEMIAS

Myeloid leukemia, chronic. (Pl. IV, Fig. 3.)

Red cells.—In the later stages of chronic myeloid leukemia with marked anemia, the red cells may show all of the alterations seen in pernicious anemia; megaloblasts may be found.

White cells.—Generally, there is a great increase in the number of white corpuscles (even to 1,000,000 or more), the blood picture being characterized by the large number of immature corpuscles from the bone marrow, i.e., *myelocytes,* neutrophilic, eosinophilic, and basophilic, and *myeloblasts.* There is greater variation in size of the leukocytes than one encounters in normal blood. The blood film resembles a smear of bone marrow.

Platelets.—No special change.

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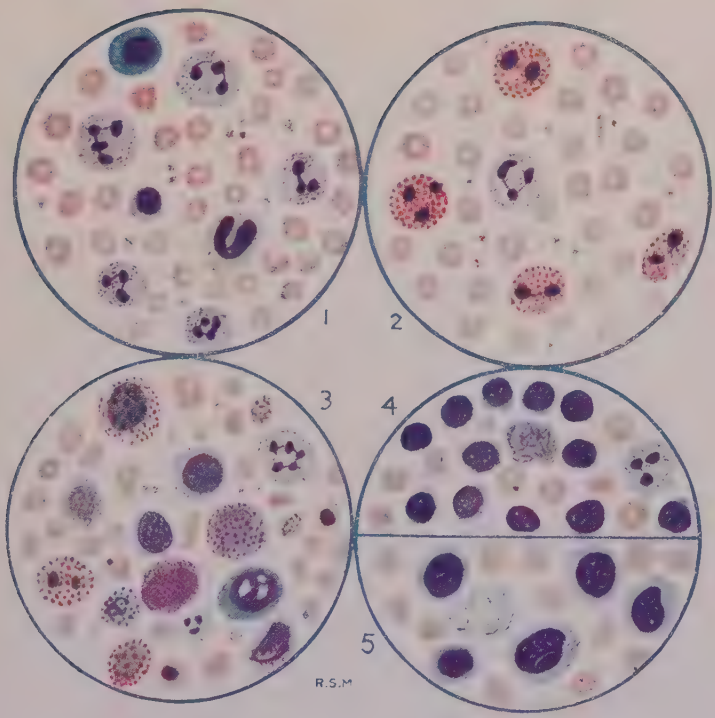
[60] For discussion of polycythemia see F. Parkes Weber. "Polycythemia, Erythrocytosis and Erythraemia." *Quarterly Jour. of Med.,* 1908-9, 11, 85-134; *Polycythaemia, Erythrocytosis and Erythraemia (Vaquez-Osler disease),* London, 1921.

The count of 15,900,000 occurred in a patient on the Medical Service of the Cincinnati General Hospital (1922). The count was made, and controlled, by Dr. Raphael Isaacs, using U. S. Bureau of Standards hemocytometer and pipettes.

Legend for Plate IV

(Wright's stain; ×800.)

1. Neutrophilic leukocytosis. (The mononuclear, with very basophilic, vaculoated cytoplasm is a Türk's irritation form.)
2. Eosinophilia.
3. Chronic myeloid leukemia.
4. Chronic lymphoid leukemia.
5. Acute lymphoid leukemia (showing a "shadow" or "basket" cell, the remnant of a lymphocytic nucleus).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Myeloid leukemia, acute.

Red cells.—There is a rapidly advancing anemia, often of the aplastic type (that is, without evidences of regeneration).

White cells.—The leukocyte count may be normal; more often it is increased, at times to several hundred thousand per c. mm.; occasionally, there is a leukopenia. The differential count is usually characterized by a high percentage of myeloblasts, the nongranular antecedents of the myelocytes. In some cases, the percentage is ninety or more, leading to great difficulty in differentiating between this and acute lymphoid leukemia; however, myelocytes and promyelocytes (p. 307) are usually conspicuous and serve to differentiate.

Platelets.—Decreased. *Bleeding time* prolonged.

Lymphoid leukemia, chronic. (Pl. IV, Fig. 4.)

Red cells.—There is a secondary anemia, often of the chlorotic type.

White cells.—They are usually greatly increased in number, to 1,000,000 or more, with a striking increase in *lymphocytes,* usually to ninety per cent and more. The predominating cell is usually the *small* lymphocyte. Naked nuclei are not uncommon. The nuclei are fragile, and many are broken in making the smear, as in all leukemias.

Blood platelets are usually decreased in number.

Lymphoid leukemia, acute. (Pl. IV, Fig. 5.)

Red cells.—There is usually a rapidly advancing anemia of the aplastic type.

White cells.—They are usually increased in number, though the count may remain normal or subnormal. The predominating cell is generally a *large* lymphocyte. Cells with irregularly shaped nuclei (Rieder cells, q. v.) may be numerous.

Platelets are markedly decreased in number. *Bleeding time* prolonged.

NEUTROPHILIC LEUKOCYTOSIS [61]

Neutrophilic leukocytosis (Pl. IV, Fig. 1). (Polynuclear neutrophiles normally 65 to 70 per cent, or 3,200 to 7,000 cells per c. mm.).

Physiological.—Leukocytosis may be physiological during the later

[61] Modified after Naegeli, O. *Blutkrankheiten und Blutdiagnostik,* 2d ed., 1912, p. 297.

weeks of (1) *pregnancy* and during *labor;* after (2) [62] *severe physical exertion;* and during (3) *digestion* (inconstant and slight).

Pathological.

Infectious diseases with neutrophilic leukocytosis.

1. *Pneumonia* and pneumococcus infections.
2. Acute cerebrospinal *meningitis.* (Meningococcus, pneumococcus, streptococcus, etc.)
3. *Diphtheria.*
4. *Septic infections* (so long as they are active and progressive).
5. *Erysipelas.*
6. *Acute rheumatic fever.*
7. *Scarlet fever.*
8. *Small-pox* (about the eighth day, then a slight decline, with a secondary rise the twelfth to fourteenth day).
9. *Cholera.*
10. *Lethargic encephalitis* (inconstant).
11. *Acute anterior poliomyelitis.*
12. *Plague* (up to 100,000 +; often leukopenia in septicemic form).[62]
13. *Typhus fever* [62] (inconstant).[63]
14. *Yellow fever* (inconstant).[62]
15. *Verruga peruviana.*[62]
16. *Relapsing fever.*
17. *Trench fever* (inconstant) (Swift).[64]
18. *Post-malarial.*

Infectious diseases without leukocytosis.

1. *Typhoid fever.*
2. *Paratyphoid fever.*
3. *Measles.*
4. *Mumps.*
5. *Influenza.*
6. *Tuberculosis.*
7. *Pertussis* (lymphocytosis) (total count increased).

Leukocytosis in these diseases indicates a complication. Often, the total count remains within normal limits, but a percentage increase of the neutrophiles is found.

[62] Castellani, A., and Chalmers, A. J. *Manual of Tropical Medicine,* 3d ed., 1919.

[63] Sellards, A. W. "Typhus Fever, with Particular Reference to the Serbian Epidemic." *The American Red Cross.* Cambridge, Mass., 1920.

[64] Swift, H. F. "Trench Fever. Report of Commission." *Medical Research Committee, American Red Cross,* 1918.

Neutrophilic leukocytosis also occurs in:

19. *Intoxications.*—Turpentine, digitalis preparations, camphor, etc., when given subcutaneously; bichloride of mercury poisoning (H. B. Weiss).
20. *Beri-beri.*
21. After severe *hemorrhage.*
22. With *malignant disease,* especially when metastases involve the bone marrow. (In a recent case of carcinoma of the rectum in the Medical Clinic of the Cincinnati General Hospital, with very extensive metastatic involvement of the liver and without bone involvement (autopsy), there were 99,300 leukocytes per c. mm. with 97 per cent polynuclear neutrophiles.)
23. *Agonal* (inconstant).

EOSINOPHILIA

Eosinophilia (Pl. IV, Fig. 2).—(Eosinophiles normally 2 to 4 per cent, or 100 to 400 cells per c. mm.) An eosinophilia, like a neutrophilic leukocytosis, may be *relative* or *absolute;* often the two coexist. An example of a relative eosinophilia is observed not infrequently in pernicious anemia, where, with a leukopenia, there may be a normal number of eosinophile cells per c. mm., with a relative (that is, a percentage) increase of these cells. If the absolute number of cells per c. mm. is above normal, there is then an absolute, as well as a relative, eosinophilia.

A physiological eosinophilia has not been described.

Eosinophilia may be found in:

1. *Bronchial asthma* and *hay fever* during and shortly after the paroxysm.
2. Other *anaphylactic states.*
3. *Animal parasitic infections.*
4. Certain *skin diseases,* as urticaria, psoriasis, pemphigus, dermatitis herpetiformis, etc.
5. Chronic *antimony poisoning* (Schrumpf and Zabel).
6. At times in *scarlet fever* (Nægeli).
7. During *convalescence* from many *acute infections.*
8. During periods of *blood regeneration* at times.
9. *Myeloid leukemia.*

10. Generalized *carcinomatosis* and *lymphogranuloma* at times (Strisower).
11. *Endocrine disturbances* (inconstant). (Borchardt; Bence and Engel.)
12. Generalized *blastomycosis* (Naegeli).
13. Certain cases of *proctitis* and *colitis* (amebic to 20 per cent).
14. *Sprue* (Castellani and Chalmers).

LYMPHOCYTOSIS

Lymphocytosis (Pl. IV, Figs. 4 and 5).—(Lymphocytes normally 20 to 25 per cent; or 1,500 to 2,500 cells per c. mm.) Lymphocytosis is, like eosinophilia, frequently unassociated with an increase in the total number of cells. With a normal count, a percentage increase leads to an actual increase in the total number of cells per c. mm. of blood. With a leukopenia, the increase may be merely *relative,* that is, an increased percentage, but the total number of cells per c. mm. remains normal or may be even subnormal. *Examples:* (*a*) *Relative* lymphocytosis. Total leukocyte count 4,000; lymphocytes 50 per cent; lymphocytes per c. mm. 2,000. (*b*) *Absolute* lymphocytosis. Total leukocyte count 14,000; lymphocytes 35 per cent; lymphocytes per c. mm. 4,900.

The following are among the more important causes of lymphocytosis:

a. Physiological.—Children up to ten years of age, 40 to 60 per cent (up to 5,400 lymphocytes per c. mm. or, rarely, more).

b. Pathological.

1. *Lymphoid leukemia.*
2. *Aleukemic lymphadenoses.*
3. *Whooping-cough.*
4. *Post-infectious.*
5. *Post-toxic;* following *serum disease* (Naegeli).
6. *Syphilis* during secondary eruptive stage (at times, increase in large mononuclears).
7. *Hyperthyroidism.*
8. Some cases of *nontoxic goiter,* and *hypothyroidism.*
9. *Dyspituitarism* (inconstant).
10. *Obesity* (Caro).
11. *Addison's disease* (inconstant).
12. *Pernicious anemia* (relative).

13. *Diabetes mellitus* (inconstant).
14. *Focal infection* with *Streptococcus hemolyticus* or *S. viridans* (inconstant) (Daland).
15. *Pulmonary tuberculosis,* before secondary infection occurs.
16. *Chronic polyarthritis* (Gudzent) (*Vide* 14).
17. Repeated exposure to the *X-rays.*
18. *Pellagra.*
19. *Rickets.*
20. *Scurvy.*
21. *Banti's disease* (inconstant).
22. *"Infectious mononucleosis."* [65] (Increase of small and large, atypical lymphocytes with fever, swelling of lymph glands and spleen, suggesting leukemia.)
23. *Septicemia,* severe (inconstant). (In a recent case, white count two days before death 700 cells per c. mm.; lymphocytes 90 per cent.)

MONONUCLEOSIS

Mononucleosis.—(The large mononuclears, including the so-called "transitionals," which probably represent later stages of the mononuclears, constitute normally about 5 to 8 per cent of the leukocytes, or 250 to 800 cells per c. mm.)

The following are the more important causes of mononucleosis:

1. *Typhoid fever.*
2. *Malaria,* during the afebrile periods (up to 90 per cent),[66] persisting for months after the fever has subsided.
3. *Leishmaniasis* (up to 70 to 80 per cent, with leukopenia).[67]
4. *Amebic dysentery* (chronic type).[67]
5. *Malta fever* (up to 80 per cent).[67]
6. *Rocky Mountain spotted fever.*
7. *Pellagra.*

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[65] Sprunt, T. P. and Evans, F. A. "Mononuclear leucocytosis in reaction to acute infections. ("Infectious mononucleosis.") *Johns Hopkins Hosp. Bull.,* 1920, XXXI, 410.

[66] Thomson, D. "The leukocytes in malaria; a method of diagnosing malaria long after it is apparently cured." *Ann. Trop. Med. and Parasitol.,* 1911, V, 83.

[67] Castellani, A., and Chalmers, A. J., *Manual of Tropical Medicine* (3d ed.), N. Y., 1919.

8. *Yaws.*
9. *Verruga peruviana* during the decline of the disease (neutrophilic leukocytosis during the early stages).[67]
10. *Trypanosomiasis.*
11. *Dengue.*
12. *Pappataci fever.*[67]
13. *Tertiary syphilis* (inconstant).
14. *Sprue.*
15. *Blackwater fever.*
16. *Hodgkin's disease.*[68]
17. *Tetrachlorethane poisoning.*[69]

LEUKOPENIA

Leukopenia.—An abnormal decrease in the number of leukocytes exists when the count falls below 5,000 cells per c. mm. It may be met with in the following conditions:

1. *Inanition.*
2. *Malaria.*
3. *Typhoid fever.* (*Typhoid vaccine* after 3d dose, often persisting for months.) [70]
4. *Paratyphoid fever.*
5. *Influenza* (including "influenzal pneumonia").
6. *Acute streptococcus* and *staphylococcus infections* at times. (In a recent case of Streptococcus septicemia (autopsy), the leukocytes fell to 700 per c. mm., 90 per cent of the cells being lymphocytes.)
7. *Chronic streptococcus infections; focal streptococcic infections* at times, especially when active (Daland).
8. *Measles.*
9. *Pernicious anemia.*
10. *Aplastic anemia.*
11. *Acute leukemias* (rather uncommon, usually increased count).

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[68] Bunting, C. H. "The blood picture in Hodgkin's disease." *Johns Hopkins Hospital Bull*, 1914, XXV, 173.

[69] Minot, G. R. and Smith, L. W. "The blood in tetrachlorethane poisoning." *Arch. Int. Med.*, 1921, XXVIII, 687.

[70] Schneider, F. "Ueber Leukopenie und Anosinophilie nach Typhusschutzimpfungen." *Deutsche med. Wchnschr.*, 1915, XLI, 393.

12. Extensive *tuberculosis of the lymphatic glands* (Naegeli).
13. Prolonged exposure to *X-rays.*
14. *Poisons,* such as benzol; arsenic (in a recent case in the Cincinnati General Hospital, the white count dropped to 500 cells per c. mm. following arsphenamine intravenously); chronic alcoholism; antimony.
15. *Banti's disease.*
16. *Leishmaniasis.*
17. *Dengue.*
18. *Typhus fever* (inconstant).

SPIROCHETES

1. **Treponema novyi** (Syn.: *Spirochaeta novyi, Spiroschaudinnia novyi*).—This was identified by Novy in 1906, and is the causative agent of *North American relapsing fever.* The spirochetes are 8 to 20 micra in length, 0.25 micron thick. There are 6 to 8 waves in the spiral. The parasites are very numerous in the peripheral blood during the febrile periods, and may be demonstrated by direct microscopic examination, by dark field illumination or by staining the blood. The organisms are very actively motile.

2. **Treponema recurrentis** (Syn.: *Spirochaeta recurrentis, S. obermeyeri, Spiroschaudinnia recurrentis*).—This was discovered by Obermeyer, and is the causative agent in *European relapsing fever.* It is found in the blood in short and long forms. The short forms are 7 to 9 micra long, the long forms 16 to 19 micra. The width is 0.25 micron. Agglutination of two or more spirochetes may lead to forms 18 to 100 micra long (Castellani and Chalmers). The short form has a long flagellum at one end (Novy and Knapp). The spirochetes are numerous, and are found by direct microscopic examination of the blood, by dark field illumination or by staining. They are actively motile.

3. **Treponema icterohemorrhagiae** (Syn.: *T. icterogenes, Spiroschaudinnia icterohemorrhagiae, Spirochaeta icterohemorrhagiae, Leptospira icterohemorrhagiae*).—This was discovered by Inada and Ido in Japan in 1913, and is the causative agent of *spirochetal jaundice* (infective jaundice, icterus castrensis gravis, Weil's disease). The spirochetes are 6 to 9 micra long as a rule, but may reach a length of 20 micra; they are about 0.25 micron in thickness. There are two or three small, or four or five large waves. At times, there are 24 to 34 small waves in the

spiral. A few spirochetes may be found *in the blood* between the fourth and the ninth days of the disease (difficult to see) and *in the urine.* The cerebrospinal fluid is infectious.

To detect the spirochetes in the urine (they are demonstrable after the first week), Castellani recommends centrifuging 20 c.c. of urine, pouring off the supernatant fluid, then adding another 20 c.c.; this is continued until about 200 c.c. have been centrifuged, when the sediment is examined. The spirochetes may be demonstrated either by dark field illumination or by staining. The urine to be examined should be obtained by catheterization; the specimen should be collected in a sterile flask.

4. **Treponema icteroides** (Syn.: *Leptospira icteroides*).—This was found by Noguchi in 1919, and is probably the cause of *yellow fever.* Morphologically, it is practically identical with *T. icterohemorrhagiae* It is found at times by direct examination of the blood and of the liver of patients ill with yellow fever, also in the blood, liver and kidneys of guinea-pigs inoculated with the blood of these patients. It has been obtained in pure culture. Noguchi succeeded in one of six trials in transmitting the disease from man to the guinea pig by means of *Stegomyia fasciata,* the mosquito vector of the disease, and in three of seven trials he has transmitted the disease from guinea pig to guinea pig by the same means.[71]

PROTOZOA IN THE BLOOD

The blood protozoa which are of pathological importance are few in number. At the present time the plasmodia of malaria alone demand general consideration in this country. For protozoa in general, however, such as trypanosomes, Leishman-Donovan bodies, etc., the method of demonstration in the stained specimen, which is universally employed, is one of the numerous modifications of the Romanowsky stain (q. v.), though often the examination of the fresh blood is even more satisfactory.

MALARIAL PARASITES

The *plasmodia of malaria* (see Pl. V, and also table on p. 332 and 334) are characteristically stained by the Romanowsky method. The *protoplasm* (Pl. V) of the parasite is stained light blue, contrasting well with the pink color of the red corpuscle. The *nuclear chromatin* of the

[71] Neveu-Lemaire, M. *Précis de Parasitologie humaine.* Paris, 1921, 5th ed., p. 150.

parasite is colored a brilliant red or purplish red, while the *pigment* retains its original color, being unstained.

In *certain* of the infections with *Plasmodium vivax* and *Plasmodium falciparum,* peculiar granulations appear in the infected red corpuscles (Schüffner's granules). They have been described by Schüffner and others. With Romanowsky stains, the granules exhibit a dark, reddish tint, often quite like that of the chromatin of the parasite. *Schüffner's granules* (Pl. V (A), cell 5) are not to be confused with the ordinary basophilic granules of the red cells or with the red granulations seen in certain corpuscles when stained with Romanowsky stains. By means of vital staining Boggs [72] has adduced further proof of the nonidentity of Schüffner's with other granules. The granules may be missed in cells containing the youngest hyalin parasites, and usually seem to increase in number with the age of the parasite. Schüffner's granules have not been observed in cells infected with the parasite of quartan fever.

Blood platelets have been mistaken for hyalin forms of the plasmodia by inexperienced observers. This is apt to occur only when the platelet rests upon the red corpuscle (Pl. V (A), (B), cell no. 1). Differentiation is simple. The chromatin of the platelet is usually colored purple with less of the reddish tint than the chromatin of the parasite shows, but this difference may be lacking, for often the chromatin of the parasite is stained exactly the shade of that of the platelet. The important differential point is found in the arrangement of the chromatin. In the platelet the chromatin is scattered in minute granules, while the chromatin of the *hyalin* parasite is in a compact mass, or, in the case of *Plasmodium falciparum,* in two or three masses, but still dense and compact. The body of the platelet is often unstained, but may take a pale blue color, very much like that of the protoplasm of the parasite. Giant blood platelets have been mistaken for extracellular forms of the malarial parasite. The constant presence of pigment granules in the larger forms of the parasite should be sufficient to differentiate, even though the distribution of the chromatin in this instance be somewhat similar in the two—which is generally not the case.

Examination of the Fresh Blood (see table, p. 332).—Whenever possible, examination of the fresh blood is the most satisfactory

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[72] Boggs, T. R. "Vital staining of 'stipple cells' in malarial blood." *Jour. A. M. A.,* 1911, LVII, 150.

THE APPEARANCES OF MALARIAL PLASMODIA IN FRESH BLOOD

	Pl. vivax (*tertian*)	*Pl. falciparum* (*estivoautumnal*)	*Pl. malariæ* (*quartan*)
I. Hyalins.			
a. Shape.	Often irregular, occasionally ring forms.	Usually ring forms, occasionally irregular.	Irregular or ring forms.
b. Refractivity.	*Difficult to see;* much like the red cell. Ring forms more refractive.	*Easily seen;* refractive.	Rather easily seen.
c. Motility.	Actively ameboid.	Occasionally active. Rings sluggish.	Sluggish usually.
d. Multiple infections.	Infrequent.	*Frequent.* May be six or more hyalins in a single cell.	Infrequent.
II. Pigmented forms.	Ameboid, very irregular. Three-quarters and full grown parasites round.	*Usually no pigmented asexual forms in circulating blood.* Round or oval, when seen.	Irregular, soon becoming round or oval. "Band" forms not infrequent.
a. Shape.			
b. Refractivity.	Young forms *difficult to see.*	*Easily seen;* refractive.	*Easily seen.*
c. Motility.	Young forms *actively ameboid.*	Sluggish.	Sluggish.
d. Pigment.	Fine brown granules scattered *throughout parasite.*	Fine, dark brown granules, *centrally placed.*	Coarse brown granules, *peripherally placed.*
e. Motility of pigment.	*Very active in younger forms.*	Sluggish.	Very sluggish.
f. Merozoites or daughter parasites.	12 to 24, usually about 16.	8 to 24, usually 12 to 16, small.	*6 to 12, often 8.*
III. Sexual forms. Shape.	Round.	Usually *crescentic,* at times round or oval.	Round.
IV. Infected red cells.	*Swollen and pale.*	Often brassy, shrunken or crenated.	Often brassy, *no swelling.*

method of diagnosis of malaria, as the variety of the parasite is more easily recognized, as a rule, than in stained smears. The accompanying table gives the main differences between the three species of plasmodia in fresh blood; it may be used also, with certain obvious exceptions, in connection with stained smears.

Degenerations in the red cells may be mistaken for hyalin parasites. In form, the degenerations may bear a striking resemblance to ring forms or irregularly shaped parasites. Ameboid activity is lacking, and it may be noted that the degenerations become more numerous in the specimen as time advances. The larger round or oval degenerations are less confusing; their size appears to change on raising and lowering the focus.

Staining Method of Ross.[73]—Ross has devised a method for detecting the parasites which is useful when their number in the blood is small. He prepares a thick smear of the blood and, before staining the film, extracts the greater part of the hemoglobin from the cells. A drop of blood of about 20 c. mm. is placed on a cover glass (¾ in. square) and spread in the usual manner, or with a needle or lancet. It is dried in the air. The preparation contains about twenty times the amount of blood usually found in a smear. After becoming dry it is covered with 1 per cent eosin solution (Romanowsky's). The stain is placed on the specimen with a glass rod and is allowed to act for as much as fifteen minutes. It is then washed. The washing must be done with a very gentle stream, since the unfixed blood is easily loosened. Then stain with Romanowsky's methylene blue[74] a few seconds and again wash carefully. The specimen is dried in the air and mounted in balsam. The hemoglobin is extracted from the red corpuscles, leaving only their stromata, together with leukocytes, platelets, and plasmodia. The staining of the parasite is distinctive—protoplasm light blue and nuclear chromatin red. Hyalins are readily detected.

For finding larger, pigmented parasites Ross prepares the film as described above; after it has dried, it is covered with distilled water to extract the hemoglobin. The unstained specimen is then examined. Crescents and other pigmented forms stand out prominently.

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[73] Ross, R. "An improved method for the microscopical diagnosis of intermittent fever." *Lancet*, 1903, I, 86.

[74] Nocht's solution of methylene blue for the Romanowsky stain consists of methylene blue (rectif. puriss. Hoechst) 1.0 gm., sodium carbonate 0.5 gm., dissolved in 100 c.c. of distilled water.

DIFFERENTIAL TABLE OF MALARIAL PLASMODIA WITH ROMANOWSKY STAINS

	Plasmodium vivax	*Plasmodium falciparum*	*Plasmodium malariæ*
Hyalins	Stains red; chromatin in single mass.	Stains red; *chromatin often rod-like or in two or three masses.*	Stains red; chromatin in single mass.
Nucleus.			
Cytoplasm	Pale blue.	Pale blue.	Pale blue.
Shape	Rings and irregular forms.	Rings usually.	Rings and irregular forms.
Multiple infections.	Infrequent.	*Frequent.*	Infrequent.
Pigmented Forms			
Pigment:			
First appears	*6 hours after chill.*	*After parasite is 24 hours old.*	*10 hours after chill.*
Distribution	*Scattered throughout.*	*Central.*	*Peripheral.*
In peripheral blood	*Present.*	*Absent*, except in very severe infections.	*Present.*
Infected Red Cell			
Size	*Swells* after 18 hours.	Normal or shrunken; crenated at times.	Normal.
Color	Pale after 18–24 hours.	May be darker.	Often darker.
Schüffner's granules	Present at times.	Present at times.	*Not found.*
Daughter Parasites			
Number	12 to 14, usually about 16.	8 to 24, usually 12 to 16.	*6 to 12, often 8.*
Arrangement	Regular.	Irregular. (Rare in peripheral blood.)	Regular, *rosette common.*
Macrogametes.			
Shape	Round or oval.	*Crescentic;* occasionally round or oval.	Round or oval.
Size	Larger than mature schizont.	9–14 micra long, 2–3 thick.	Larger than mature schizont.
Nucleus	Small, peripheral.	Small, at middle of crescent.	Small, peripheral.
Cytoplasm	Deeper blue.	Deep blue.	Deep blue.
Pigment	Heavy, scattered.	Collected about nucleus.	Heavy, peripheral.
Microgametocytes			
Shape	Round or oval.	*Crescentic*; plumper than former.	Round or oval.
Size	Smaller than macrogamete.	Shorter and thicker than macrogamete.	Smaller than macrogamete.
Nucleus	Large, central.	Large, occupying much of crescent.	Large, central.
Cytoplasm	Pale blue.	Pale blue.	Pale blue.
Pigment	Heavy, scattered.	Chiefly at poles of crescent.	Peripheral, heavy.

Ruge's Modification of the Method of Ross.[75]—A disadvantage in the method of Ross is the difficulty of washing the unfixed specimen without losing the preparation. To overcome this, Ruge fixes the thick films in 2 per cent formalin containing ½ to 1 per cent acetic acid. The hemoglobin is extracted from the erythrocytes, which are fixed to the cover glass at the same time. The specimen is then stained with Romanowsky's stain. The formalin fixation interferes somewhat with the staining of the protoplasm of the plasmodia, so that it may be necessary to restain the film with methylene blue. A certain amount of precipitate remains in the specimen, but the parasites are well stained.

THE MALARIAL PARASITES IN THE STAINED BLOOD (ROMANOWSKY STAIN)

Plasmodium Vivax (Pl. V, *A*).—The parasite of *tertian fever* requires forty-eight hours for the complete development of the asexual forms (Fig. 81). The parasites of a given group all mature at about the same time, and clinically this is manifested by a chill. With a *single* group of tertian parasites, the patient has a chill every other day at about the same hour, while with a *double* tertian infection there is a daily chill (quotidian fever), and two groups of parasites are found in the blood. At the time of the chill, one finds in the blood segmenting parasites and the young (daughter) forms (merozoites).

The *hyalin* parasite has a deep red nucleus, about 1 micron in diameter, the chromatin being surrounded by a clear, achromatic zone; the cytoplasm of the parasite stains pale blue. In the next few hours, ring forms appear, probably due to the inclusion of parts of the red cell by the parasite (Pl. V, Fig. 2-*A*). The chromatin is usually found in the thinnest or narrowest part of the cytoplasm of the parasite. *Six hours* after the chill, the parasite is about 5 micra in diameter, and now the first extremely fine, brownish *pigment* granules are visible (Pl. V, Fig. 4-*A*). Now, ring forms are uncommon, the shape of the parasite being irregular. After the parasite fills about one-third of the red cell, Schüffner's granules *may* appear in the infected red cell (Pl. V, Fig. 5-*A*). (They are inconstant, being found in some cases of tertian and estivo-autumnal malaria, not in others.) At *sixteen hours*, the parasite may reach the volume of about one-third of a normal red cell.

[75] Ruge, R. "Zur Erleichterung der mikroskopischen Malariadiagnose." *Deutsche med. Wchnschr.*, 1903, XXIX, 205.

Legend for Plate V

(*A*, *B*, *C*, Wright's stain. *D*, Leishman's stain. *E*, Ziehl-Neelsen stain; ×800.)

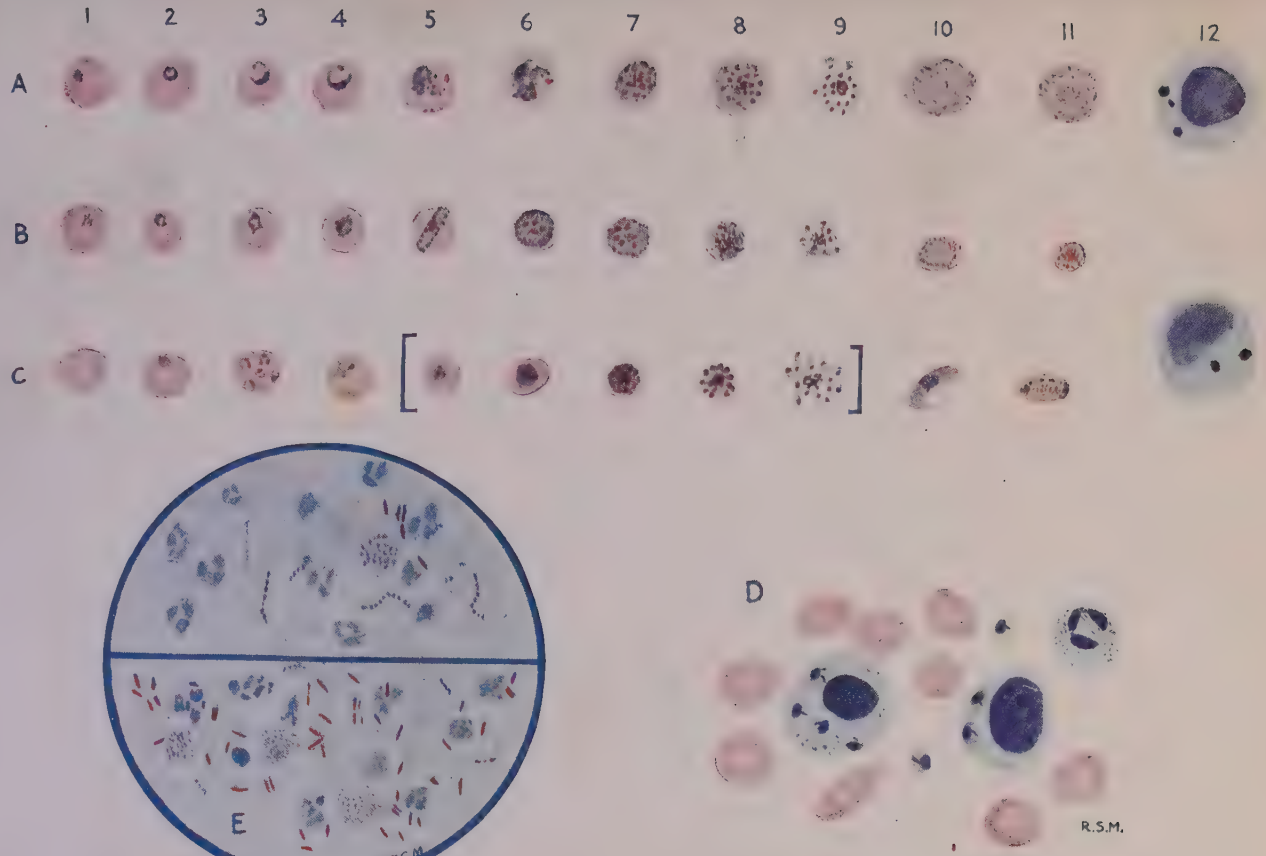
A. *Plasmodium vivax* (tertian). 1, a red cell with platelet superimposed; 2 to 4, hyalin (non-pigmented) parasites; 5 to 8, pigmented parasites; cell 5 shows Schüffner's granules in the red blood corpuscle; 9, daughter parasites or merozoites and free pigment; 10, macrogamete (the female sexual form); 11, microgametocyte (the male sexual form); 12, a large mononuclear leukocyte (macrophage) containing malarial pigment. Note swelling and pallor of infected red cells.

B. *Plasmodium malariae* (quartan). 1, a red cell with blood platelet superimposed; 2, 3, hyalin (non-pigmented) parasites; 4 to 8, pigmented parasites; 5 is a band form of the parasite; 9, daughter parasites or merozoites and free pigment; 10, macrogamete (the female sexual form); 11, microgametocyte (the male sexual form). Note absence of swelling and pallor of infected red cells.

C. *Plasmodium falciparum* (syn.: *Laverania malariae*) (aestivo-autumnal, malignant tertian, subtertian) 1, a normal red cell; 2 to 4, hyalin (non-pigmented) parasites; 3, multiple infection of an erythrocyte; 5 to 8, pigmented parasites; 9, daughter parasites and free pigment. *The forms included within the brackets are rarely encountered in the peripheral blood.* Note premature division of the chromatin of the parasite. 10, macrogamete (the female sexual form); 11, microgametocyte (the male sexual form). Note the "apron" or "bib" on the crescents, 10 and 11. 12, a large mononuclear leukocyte (macrophage) containing malarial pigment.

D. *Leishmania donovani* (Leishman-Donovan bodies) in the large mononuclear leukocytes and free in the plasma (combined field).

E. *Tubercle bacilli in the sputum*. Upper half: unselected particle of sputum taken for staining. Lower half: same specimen; the material for staining was a particle containing elastic tissue.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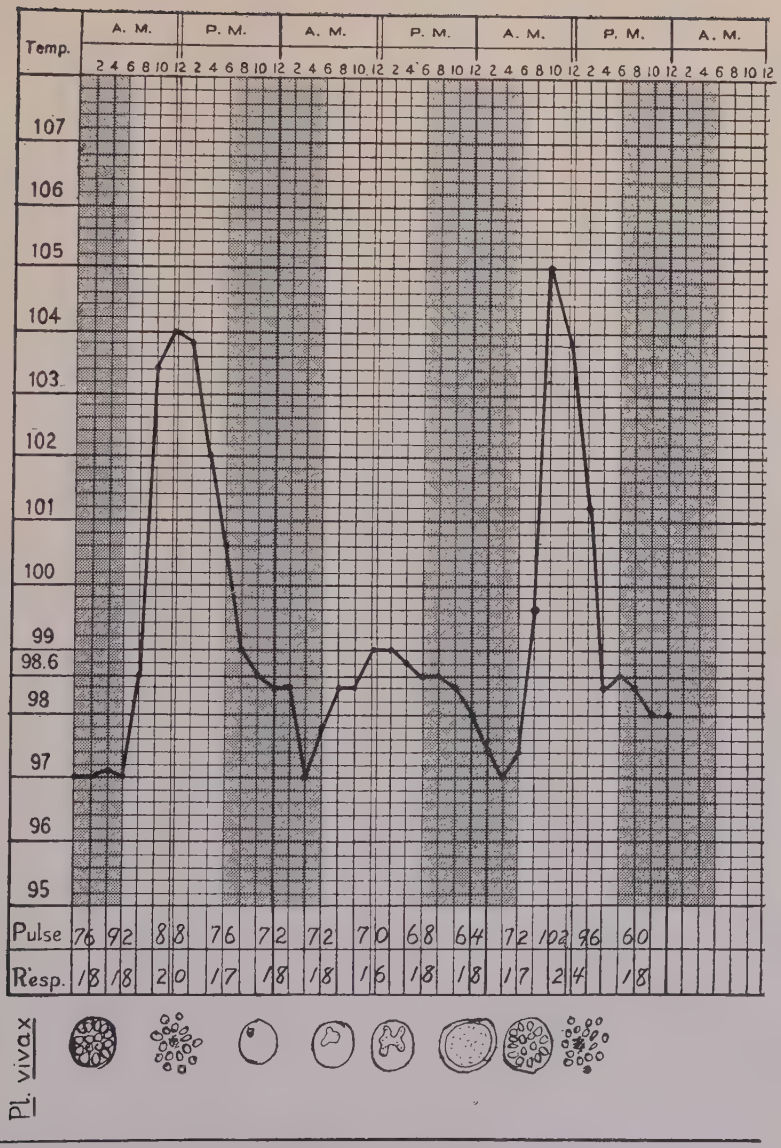

FIG. 81.—TEMPERATURE CURVE IN SINGLE (BENIGN) TERTIAN INFECTION, AND THE CORRESPONDING EVOLUTION OF *Plasmodium Vivax*.

The nucleus remains peripheral, often eccentric, as if it had no relation to the protoplasm of the parasite (Pl. V, Fig. 5-*A*). The achromatic zone is well marked. Even now, the chromatin *may* show clumping in the middle or may form closely approximated chromosomes. At *twenty-four hours,* the parasite fills one-third to one-half of the infected red cell, which has become pale and swollen. The shape of the parasite is very irregular (Pl. V, Fig. 6-*A*). The pigment granules are scattered throughout the parasite. The chromatin is usually fairly compact. At *thirty-six hours,* the infected red cell may be twice its normal size, and is pale, and the parasite fills one-half to two-thirds of it. The parasite is now generally round or oval. The chromatin has increased in volume two to three times that of the hyalin parasite, the formation of chromosomes has increased (Pl. V, Fig. 7-*A*). The red blood cell disappears at the beginning or at the completion of nuclear division. When nuclear division is complete (Pl. V, Fig. 8-*A*), the matured parasite may have an uneven outline, due to the young parasites. The pigment has now collected at the center of the parasite. Each of the new (young) chromatin masses is surrounded by a more or less evident achromatic zone and a small amount of cytoplasm derived from the mother parasite. The number of the newly formed young parasites (*merozoites*) is usually 16 (extremes 12 to 24). The size of the merozoites is 1.5 to 2.5 micra. In the same group they often differ much in size (Pl. V, Fig. 9-*A*). In one-half to one hour, they enter red blood corpuscles, and a new asexual cycle is begun (Fig. 82).

The *pigment,* which is set free in the blood plasma when the parasite segments, is taken up by the *large mononuclear leukocytes* (the macrophages) and appears as brownish masses in the cytoplasm (Pl. V, Fig. 12).

Sexual forms of the parasite (Fig. 82) make their appearance in the blood. There are certain differences between the *gametes* (sexual forms) and *schizonts* (asexual forms). The gametes are generally larger and have a tendency to be round, i. e., they show fewer ameboid pseudopodia and are decidedly less ameboid than the schizonts. The pigment in the sexual forms is heavier, coarser, and develops earlier. The chromatin becomes thinned or spread out earlier than in the schizonts, but it does not divide. The duration of the life cycle of the sexual forms is at least twice that of the asexual forms. This fact, it is said, explains the greater abundance of the pigment.

The *macrogametes* (female sexual forms) can usually be differen-

tiated from the schizonts and from the microgametocytes (males) by (*a*) the thick, granular, dark blue cytoplasm, (*b*) the slight development of the nucleus, which is nearly always peripheral and contains little chromatin, (*c*) the persistence in the blood weeks or months after the

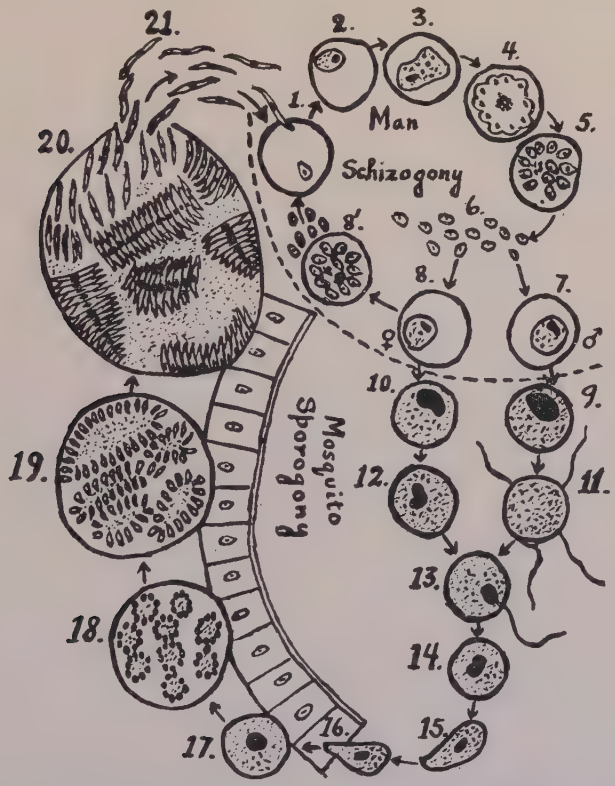

FIG. 82.—EVOLUTION OF PLASMODIUM VIVAX. Sexual and asexual cycles in the mosquito and in man. *Diagrammatic*. 1. Red cell invaded, on the one side by a merozoite, on the other by a sporozoite. 2 and 3. Schizonts. 4 and 5. Segmentation of a schizont. 6. Free merozoites. 7 to 11. Microgametocytes, 8 to 12. Macrogametes. 8^1. Regressive schizogony (parthenogenesis). 13 and 14. Fertilization. 15. Free zygote. 16. Zygote penetrating the wall of the mosquito's stomach. 17 to 20. Development of the oöcyst in the wall of the mosquito's stomach. 21. Free sporozoites (after Neveu-Lemaire).

fever has subsided, (*d*) their large size 12 to 14 micra, and (*e*) the pigment, which is abundant and more often rodlike than in the schizonts; the particles of pigment may be 1 to 3 micra long (Pl. V, Fig. 10-*A*).

The *microgametocytes* (males) are characterized by (*a*) very pale

hyalin, difficultly staining protoplasm, (*b*) a large amount of chromatin, which may fill one-third to one-half the parasite and is usually more or less centrally situated, the chromatin being to the cytoplasm of the parasite as 1:1 to 1:4 in the males, as 1:8 to 1:12 in the females, (*c*)

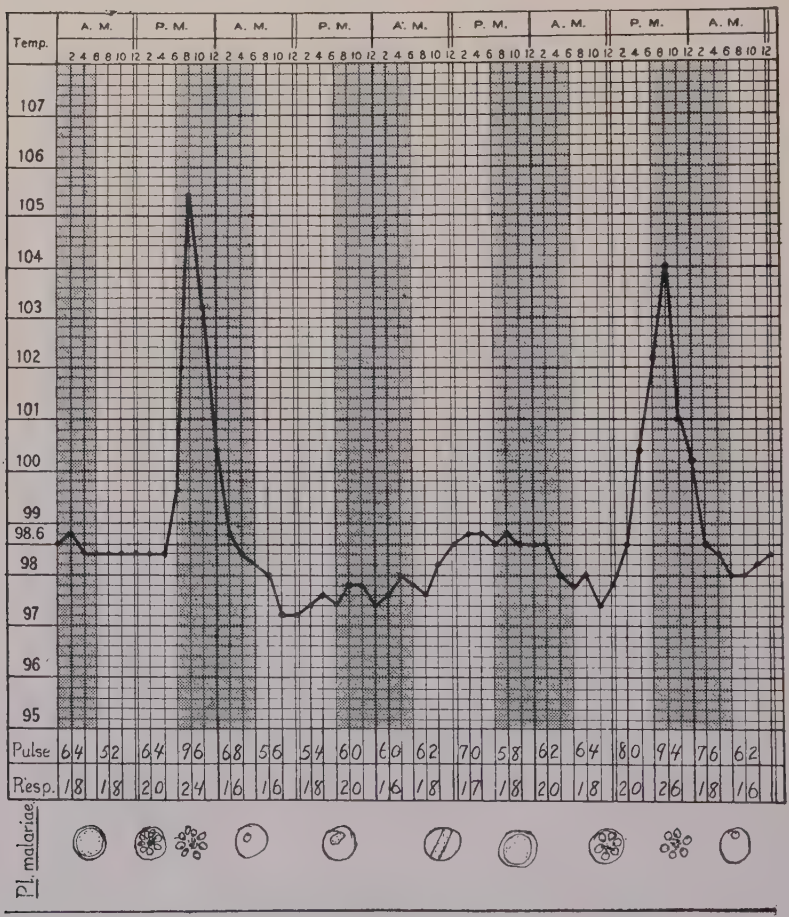

FIG. 83.—TEMPERATURE CURVE OF SINGLE QUARTAN INFECTION, AND THE CORRESPONDING EVOLUTION OF PLASMODIUM MALARIÆ.

more abundant pigment (the whirling motion of the pigment in the *fresh* blood is very characteristic), and (*d*) the size of the parasites—generally much smaller than the females, seldom larger than the schizonts (Pl. V, Fig. 11-*A*).

Plasmodium Malariae (Pl. V, *B*).—The parasite of *quartan fever* requires seventy-two hours for the development of the asexual cycle (schizonts), (Fig. 83). Like the tertian parasite, the parasites of a given group all mature at about the same time, the maturation or sporulation of the parasites coinciding with the chill. With infection with a *single* group of quartan parasites, there is a chill every fourth day (that is, every 72 hours), with a *double* quartan infection the patient has a chill on two successive days with one day of normal temperature intervening, and with a *triple* quartan infection there is a daily paroxysm of fever (quotidian fever).

The *hyalin* parasites cannot be differentiated with certainty from those of *Pl. vivax* in the stained film, but in the fresh blood (see table, p. 332) the ameboid movement is less marked, and this difference increases with the age of the parasite; this manifests itself in the somewhat more regular, round shape of the quartan schizonts as compared with the tertian (Pl. V, Fig. 2-*B*). At *ten hours,* dark brown pigment granules first appear, and they have a tendency to be distributed peripherally in the parasite (Pl. V, Fig. 4-*B*). At *sixteen hours,* the parasite tends to be round (it is almost immobile in the *fresh* blood), and fills one-fifth to one-fourth of the red cell, with peripherally placed pigment. (The cytoplasm of the parasite has a porcelain-like appearance in the *fresh* blood). At *twenty-four hours,* the parasite has become larger, contains more pigment, and fills one-fourth to one-third of the red cell. The nuclear chromatin occupies about one-eighth the area of the parasite. The infected red cells remain normal in size or shrink somewhat, and become darker, in contrast to the swelling and pallor of the corpuscles in tertian infections. At *thirty-six hours,* the parasite fills about one-half the red cell. Often band-forms are encountered (Pl. V, Fig. 5-*B*); indeed, they may be found at the 12 to 16 hour stage. From this age on, the quartan parasite shows a more faintly staining chromatin than the tertian. The chromatin has increased in volume two to three times. (In the *fresh* blood, forms found now with actively motile pigment granules are gametes). At *forty-eight hours,* chromatin division may, though it does not necessarily, begin, similar to the tertian parasite. The parasite is round, and only a narrow rim of the unenlarged or slightly shrunken red cell remains (Pl. V, Fig. 6-*B*). The pigment is usually distributed about the periphery of the parasite. At *sixty hours,* the red cell has often disappeared. The parasite is about the size of a normal red cell, is round and has abundant, dark

brown pigment, often collecting along radii toward the center of the parasite, the so-called marguerite forms. Nuclear division is now active (Pl. V, Fig. 8-*B*). At *seventy-two hours,* segmentation occurs, the pigment is collected in the center or in a star-like arrangement, and grouped about it are the merozoites, 6 to 12 in number, often 6 to 8 (Pl. V, Fig. 9-*B*). The daughter parasites then enter red blood cells, and a new asexual cycle is begun. Schüffner's granules in infected red cells are not found in quartan infections.

The *pigment,* set free in the plasma at the time of segmentation, is taken up by the large mononuclears. *Pigmented leukocytes* are diagnostic of malaria, though, of course, the species of the parasite cannot be determined from them (Pl. V, Fig. 12).

Sexual forms are found, as with the tertian parasite. The gametes of *Pl. malariae* have practically the same characteristics as those of *Pl. vivax* (see p. 338), except that they are smaller. *The macrogametes* are 10 to 12 micra in diameter (Pl. V, Fig. 10-*B*), the *microgametocytes* still smaller (Pl. V, Fig. 11-*B*).

Plasmodium Falciparum (Syn.: *Laverania malariae*).—The *subtertian* or *estivo-autumnal* parasite requires from thirty-six to forty-eight hours for the maturation of the asexual cycle (schizonts) (Fig. 84). When a single group of parasites is present, the patient has a chill every other day; with two groups, there is a quotidian fever. When there are several broods of parasites, as not infrequently happens with *Pl. falciparum,* sporulation, instead of occurring every other day or daily, will be at irregular intervals, the paroxysms will overlap, and the result will be an irregular type of fever. The latter is the commoner event.

The *hyalin* forms are present in varying numbers; in some cases no parasites are found in the peripheral blood, necessitating hepatic or splenic puncture for diagnosis. The parasites are small, generally ring-shaped organisms, usually situated in the periphery of the red cell. (In the *fresh* blood [see table, p. 332], they are more refractive than the tertian hyalins, and have more or less ameboid activity.) The diameter of the rings is 1.5 to 3 micra. The achromatic zone about the chromatin is not well seen. The chromatin is about ¾ micron in diameter, is often drawn out in a line, unlike that in the tertian and quartan hyalins, and may form two or three small round chromatin masses (Pl. V, Fig. 3-*C*). When there are two chromatin granules, they are often at opposite sides of the ring, or placed close to one another, giving an appearance suggesting a horse-shoe; with three masses of chromatin,

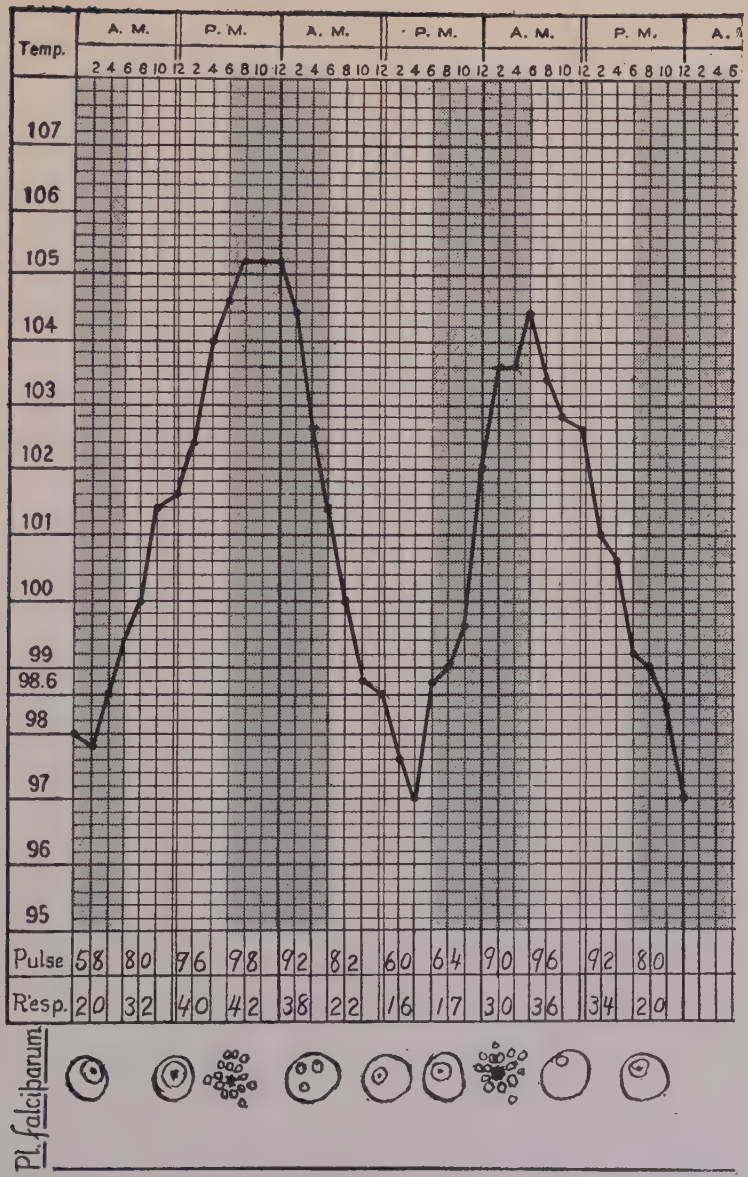

FIG. 84.—ESTIVO-AUTUMNAL OR MALIGNANT TERTIAN FEVER WITH PAROXYSMS ABOUT 38 HOURS APART, AND THE CORRESPONDING EVOLUTION OF PL. FALCIPARUM.

they may be situated about equally distant from one another about the ring. One red cell may be infected with 2 to 6 hyalins; the parasites do not fuse. *The frequency of premature division of the chromatin and of multiple infections of the red cell are characteristic, and differentiate the estivo-autumnal parasites from tertian and quartan organisms.* In addition to ring forms, one sees irregular forms (Pl. V, Fig. 4-*C*). After about *twenty-four* hours, the protoplasm gradually collects in excess at one part of the ring, forming the so-called signet ring forms. The chromatin is now about 1 micron in diameter, and the achromatic zone is well seen. *Pigment* may now begin to form in the thick part of the signet ring, and the latter, as well as the irregular forms, tend to become round. The pigment collects in the neighborhood of the chromatin. After about *thirty hours,* the *majority of the parasites leave the peripheral blood in most cases, to continue their development in the internal organs* (Pl. V, Fig. 5 to 9-*C*). In severe infections, it may be possible to follow the further development of the parasites in the peripheral blood. At *thirty-six hours,* the parasites are round or oval, and in the *fresh* blood appear as sharply defined discs, which have a ground glass appearance. The parasite fills one-fifth to one-fourth of the red cell, which now shows a tendency to shrink, to become darker, and often to crenate. The chromatin may now begin to divide, but the characteristic, very rapidly proceeding nuclear division usually begins first after the parasite has increased in size, so that it fills one-half to three-quarters of the red cell. Often one sees what appears to be a crack in the part of the infected red cell not occupied by the parasite; this is never observed in tertian or quartan infections. As the merozoites form, the red cell becomes pale and then disappears. The merozoites number 8 to 24, usually 12 to 16 (Pl. V, Fig. 9-*C*).

Pigmented leukocytes are rather commoner than with tertian and quartan infections.

Sexual forms (gametes) of the parasite are usually distinctive, and, as in the other forms of malaria, they may persist in the blood for months after the fever has subsided.

The *macrogametes* (Pl. V, Fig. 10-*C*) are usually crescents, having a thick, dark blue cytoplasm, with the nuclear chromatin in the middle of the crescent, often obscured by pigment. The crescents measure 9 to 14 micra long and 2 to 3 micra in thickness. The remnant of the red cell not infrequently may be seen projecting beyond the concavity of the crescent to form the so-called "apron" or "bib." In the *fresh* blood,

CLASSIFICATION OF THE PRINCIPAL BINUCLEATED PARASITES OF MAN *

Family	Genus	Species	Habitat
Spirochetidae Spiral Body	*Treponema.* Turns of spiral more or less short.	*T. recurrentis.*	Blood. *European relapsing fever.*
		T. novyi.	Blood. *American relapsing fever.*
		T. duttoni.	Blood. *African relapsing fever; tick fever.*
		T. vincenti.	Mouth. *Vincent's angina.*
		T. bronchiale.	Trachea, bronchi; sputum. *Spirochetal bronchitis.*
		T. ictero-hemorrhagiae.	Kidneys, urine. *Infectious jaundice.*
		T. icteroides.	Various organs. *Yellow fever.*
		T. pallidum.	Chancre: various organs. *Syphilis.*
		T. pertenue.	Various organs. *Yaws.*
Trypanosomidae Fusiform, ovoid or spherical body	*Trypanosoma.* Body fusiform; flagellated; undulating membrane.	*T. gambiense.*	Blood, spinal fluid. *Sleeping sickness.*
		T. rhodesiense.	Blood, spinal fluid. *Sleeping sickness.*
	Schizotrypanum Same as trypanosome, but at certain stages it assumes spherical form.	*S. cruzi.*	Blood; various organs. *Chagas' disease; American trypanosomiasis.*
	Leishmania. Body of variable form, spherical or ovoid. Flagellated at certain stages.	*L. donovani.*	Blood; various organs. *Kala-azar.*
		L. infantum.	Blood (rare); various organs. *Infantile splenic anemia.*
		L. tropica.	Skin. *Oriental sore.*
		L. brasiliensis.	Skin. *American cutaneous leishmaniasis; espundia.*

* From Neveu-Lemaire, slightly modified.

neither pigment nor parasite shows motility. The macrogametes are at times oval or round.

The *microgametocytes* (Pl. V, Fig. 11-*C*) have a hyalin, lightly staining cytoplasm, and the crescents are usually plumper. The pigment tends to collect at each pole, in contrast to its distribution in the female forms. The nuclear chromatin is scattered more or less over the whole body of the parasite and is more abundant than in the macrogametes. Usually, only the poles of the crescent show a definite blue color. One rarely sees round or oval microgametocytes.

LEISHMANIASIS

Leishmaniasis.—Leishmaniasis is a tropical disease rarely imported into this country. Four clinical varieties are recognized: (1) *tropical kala-azar* (tropical splenomegaly, tropical leishmaniasis, dum-dum fever) caused by *Leishmania donovani,* (2) *Mediterranean kala-azar* (infantile kala-azar, infantile splenic anemia, infantile leishmaniasis) caused by *L. infantum,* (3) *cutaneous leishmaniasis* (oriental sore, Delhi boil, Aleppo boil) due to infection with *L. tropica,* and (4) *muco-cutaneous leishmaniasis* (espundia, naso-oral leishmaniasis, American leishmaniasis) caused by *L. tropica var. americana* (*L. brasiliensis*). The first three varieties are endemic in tropical and subtropical climates in parts of the Old World, while the last has been found in South America (Peru, Brazil, Paraguay, Argentina, Colombia) and in Panama and Mexico.

The parasites (Pl. V, *E*) are indistinguishable morphologically. They are round, oval, or pyriform in shape, and measure 2 to 3.5 micra in length by 1.5 to 2 in breadth, with a granular cytoplasm. With Romanowsky stains, the cytoplasm takes a clear blue tint. There are two masses of nuclear material, a larger, spherical mass, the macro- or tropho-nucleus, which is closely approximated to the cell membrane, and a smaller micronucleus, rod-shaped, which takes the stain more intensely than the macronucleus.

In the first two varieties of leishmaniasis, the parasites may be found in the peripheral blood, especially in the first. Cummins [76] says, "The Leishman-Donovan body is to be found in the peripheral blood of nearly every case (of tropical kala-azar), provided the search is prolonged and the examination is made at a favorable moment. Rarely, the parasites are fairly numerous. It is to be remembered that the parasites are usually found in the interior of the leukocytes (that is, the large mononuclears and the polynuclear neutrophiles, rarely in the eosinophiles or in the concavity of a red cell—Castellani and Chalmers). For this reason, blood films should be so spread as to terminate in an even line before the end of the slide is reached. The bulk of the leukocytes will be collected at the terminal edge of the film, and examination will thus be greatly facilitated. About 0.3 c.c. of blood should be citrated and allowed to settle, films being carefully made, as above, from the layer

[76] Cummins, S. L. *Oxford Med.,* 1921, V, 861.

of leukocytes that forms upon the deposited red corpuscles. In all cases many films should be searched through before the peripheral blood is assumed to be free from parasites."

Castellani and Chalmers [77] describe the blood findings in *tropical leishmaniasis* as follows: "The examination of the blood is most important because, firstly, the parasite may be found in a leukocyte if carefully looked for, even in the early stages of the disease; secondly, the leukocytic changes are of the utmost importance. There is marked anemia—54 per cent of Rogers' cases giving from 4,000,000 to 2,500,000 corpuscles per c. mm.—and the hemoglobin is reduced in proportion to the erythrocytes, the color-index being normal. There is a most marked leukopenia, and Rogers reports that in 42 per cent of his cases the leukocytes were 1,000 or less, in 30 per cent 1,000 to 2,000, and in 22.6 per cent, 2,000 to 3,000. . . . There is a reduction in the polymorphonuclear neutrophiles and in the eosinophiles, and an increase in the mononuclears and lymphocytes. The diminution of the neutrophiles is thought to explain the tendency to bacterial infections. The coagulability of the blood is decreased, which explains the tendency to hemorrhage, and renders splenic puncture dangerous at times. . . . If the parasites cannot be found in the blood, an attempt may be made to find them by the examination of the exudate obtained by exciting artificial pustulation of the skin by some irritant, as suggested by Cummins. Failing this, there is puncture of the spleen or liver (preferably the latter) and withdrawal of blood for examination for parasites."

In *infantile leishmaniasis,* Castellani and Chalmers [78] say, "The blood is pale, and shows a decrease in the number of erythrocytes (1,500,000 to 3,000,000), and in the hemoglobin (below 50 per cent), which, however, is reduced in proportion to the red corpuscles; and also in the leukocytes (1,500 to 3,000), though the leukocytic formula is mononuclear (70 to 80 per cent), being especially composed of medium-sized cells. The mononuclears are increased at the expense of the polynuclears, which make up the remaining 20 to 30 per cent. There is usually some poikilocytosis and anisocytosis, but nucleated reds are rare or absent. . . . The parasites are rarely found in the blood. They may be obtained by splenic

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[77] Castellani, A. and Chalmers, A. J., *Manual of Tropical Medicine* (3d ed.), 1919, pp. 1291, 1293.

[78] *Ibid.*, p. 1301.

puncture, by liver puncture (safer), or by examination of the bone-marrow obtained by a modified trocar and cannula."

In *cutaneous leishmaniases* (oriental sore, espundia), the diagnosis is made by demonstrating the parasites in the lesion. Castellani and Chalmers [79] remove the scab from the lesion, and a scraping is taken from the floor and edges of the ulcer. The material is spread on a slide, and is then stained with Leishman's, Wright's or any of the modifications of the Romanowsky stain, and examined for the presence of *L. tropica.* The search must be prolonged in some cases, as the parasites may be very rare.

TRYPANOSOMES [80]

Trypanosoma Gambiense (Syn.: *Castellanella gambiensis, T. hominis, T. nigeriense*).—This is one of the trypanosomes causing *sleeping sickness* of Africa. It is 17 to 28 micra long and 1.5 to 2 micra in thickness.

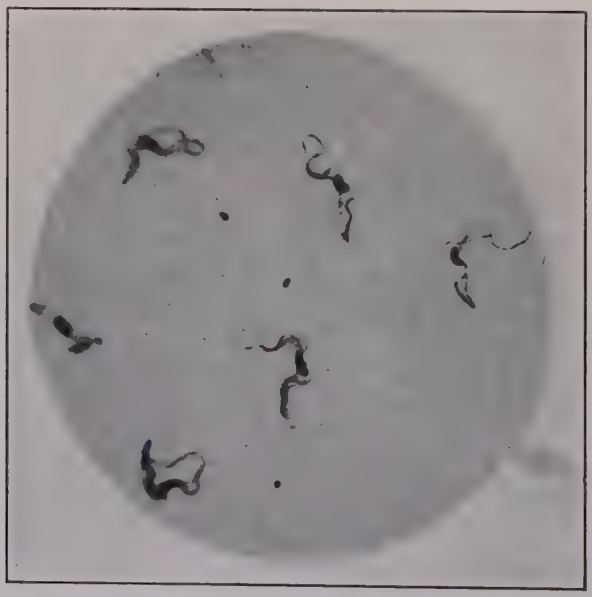

FIG. 85.—TRYPANOSOMA GAMBIENSE. ×1000 (after Wm. B. Wherry; photomicrograph by Chas. Goosmann).

[79] *Ibid.*, p. 2173.

[80] For *class demonstrations* in this country, where human material is not available, *T. lewisi*, inoculated into white rats, may be used. Often wild rats are found to be infected.

Like all trypanosomes, it possesses the following characteristics [81] (Fig. 85). The body is fusiform, and is composed of a mass of cytoplasm containing fine granules, with a large, oval nucleus, and a smaller chromatin mass, the blepharoplast. From the latter, a long flagellum extends the length of the body of the parasite, forming the edge of an undulating membrane, and becomes free at the anterior end of the parasite (Fig. 86). Man becomes infected by the bite of *Glossina palpalis,* a fly which transmits the disease. Man, antelopes, and monkeys serve as reservoirs of the virus. The organisms are found in the blood and cerebrospinal fluid of patients ill with the disease. They are actively motile and are recognized without difficulty on microscopic examination of the fresh blood, and in smears stained with one of the Romanowsky stains.

Trypanosoma Castellanii (Syn.: *T. ugandense, Castellanella castellanii*).—This is another trypanosome found in the blood and cerebrospinal fluid of patients ill with *African sleeping sickness.* It measures from 14 to 33 micra long and 2 to 2.5 micra in breadth. It is very polymorphic, having short forms 14 to 20 micra, medium forms 20 to 24 micra, and long forms 23 to 33 micra long. Morphologically, it is similar to *T. gambiense.*

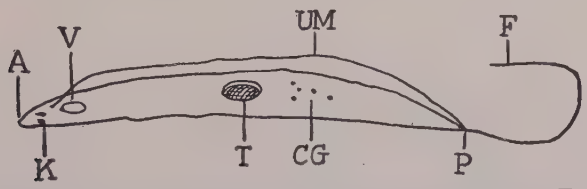

Fig. 86.—Diagram Showing the Structure and Polarity of a Trypanosome. (*A*, anterior end; *K*, kinetonucleus; *V*, vacuole; *T*, trophonucleus; *CG*, chromatin granules; *UM*, undulating membrane; *P*, posterior end; *F*, flagellum) (after Castellani and Chalmers).

Trypanosoma Rhodesiense (Syn.: *Castellanella rhodesiensis*).—This is the third of the trypanosomes causing *African sleeping sickness.* Its length varies from 12 to 31 micra. It is, like *T. castellanii,* polymorphic. Long, slender forms, 25 micra or more in length, are the most common.

Trypanosoma Cruzi (Syn.: *Schizotrypanum cruzi*).—This was discovered by Chagas in 1909 at Rio de Janeiro, and is the cause of *South*

[81] Neveu-Lemaire, M. *Précis de Parasitologie humaine.* Paris, 1921, p. 158 *et seq.*

American trypanosomiasis. In the blood of man, it resembles the trypanosomes in general, but is characterized by a terminal, voluminous blepharoplast. The young forms are slender and very motile; the larger, adult forms move slowly, and attain a length of about 20 micra. The disease affects infants particularly; in adults, it is always benign.

EXAMINATION OF THE BLOOD FOR ANIMAL PARASITES

1. **Filaria Bancrofti** (Syn.: *F. sanguinis hominis, F. nocturna; F. philippensis*).—(Fig. 87). When this parasite is present in the blood,

FIG. 87.—MICROFILARIA BANCROFTI IN THE BLOOD. Photomicrograph; ×400 (after Wherry and McDill).

it is usually demonstrable by the ordinary method of examination of the fresh blood. The size of the drop should be a little larger than usual, in order to secure a moderately thick preparation. The blood should be taken during the sleeping hours—usually at night, when the embryos

are present in the peripheral circulation. The wriggling motion of the embryos arrests attention at once. Fair permanent specimens may be secured by making a relatively thick smear of the blood, which is then stained with hematoxylin and eosin, or one of the Romanowsky stains. Stäubli's method is useful when the parasites are scarce. The embryos are 0.125 to 0.300 mm. long and 0.007 to 0.011 mm. thick (Blanchard).

2. **Loa Loa** (Syn.: *Filaria oculi; Dracunculus oculi; D. loa*).—This is a parasite of the superficial connective tissue. It is found in the conjunctiva,[82] the subcutaneous fat and the superficial aponeuroses in all parts of the body. It is endemic on the West Coast of Africa. Cases have been imported into the United States. The adult parasites may be found in the above mentioned parts of the body. The males are 25 to 34 mm. long and 0.273 to 0.430 mm. in breadth. The female is larger, measuring 44 to 63 mm. long by 0.38 to 0.49 mm. in thickness. Diagnosis is made by finding the adult worms in the subcutaneous tissues or by the finding of the embryos in the blood. The latter are present in the blood during the day but not at night, hence the designation *Microfilaria diurna*. The embryos measure 298 by 7.5 micra. They are much less numerous in the blood than those of *Filaria bancrofti*, according to Castellani and Chalmers, from whom the following table of comparison is taken in part.

Size	*Filaria bancrofti* embryos (*Microfilaria nocturna*)	*Loa loa* embryos (*Microfilaria diurna*)
Average length	0.317 mm.	0.245 mm.
Average breadth	0.0084–0.0075 mm.	0.0075–0.0070 mm.
Periodicity	In blood at night	In blood by day
Periodicity when habits of sleeping and waking are changed	Inverted	No change

3. **Trichinella Spiralis.**—The embryos of *Trichinella spiralis* are at times found in the blood. The method of detecting their presence is

[82] Begle, H. L. "Infestation with Filaria loa. Report of a case of filaria beneath the conjunctiva and microfilariae in the peripheral blood stream." *Jour. A. M. A.*, 1921, LXXVI, 1301.

that which Stäubli[83] evolved in the study of experimental trichinosis, and which has been successfully applied to the diagnosis of human infections by Herrick and Janeway[84] and others. In human cases it is apparently desirable to employ larger quantities of blood than Stäubli found necessary.

Method of Stäubli.—By means of an aspirating syringe, 1 to 10 c.c.

FIG. 88.—EMBRYO OF TRICHINELLA SPIRALIS IN BLOOD LAKED WITH 3 PER CENT ACETIC ACID. Leukocytes and disintegrated red cells are also shown; ×800 (after Herrick and Janeway).

of blood are withdrawn from an arm vein with the usual aseptic technic. The blood is immediately laked by adding it to about 15 volumes of 3 per cent acetic acid, shaking thoroughly to prevent clotting. The acid is strong enough to insure complete laking of the erythrocytes, and

[83] Stäubli, C. "Beitrag zum Nachweis von Parasiten im Blut." *München. med. Wchnschr.*, 1908, LV, 2601.

[84] Herrick, W. W., and Janeway, T. C. "Demonstration of the Trichinella spiralis in the circulating blood in man." *Arch. Int. Med.*, 1909, III, 263.

at the same time is not injurious to the embryos. The mixture is centrifugalized, the sediment removed with a pipette, and examined fresh. It consists mainly of leukocytes. The specimen should be examined with a mechanical stage, so that all parts of it may be inspected if necessary. Moderately high magnification should be employed. At the time of their entrance into the circulation the embryos (Fig. 88) are about 0.120 to 0.160 mm. long and 0.007 to 0.008 mm. in thickness (Blanchard).

Stäubli has obtained good permanent specimens by staining the sediment with Giemsa's or Jenner's stain. The acetic acid should be completely removed by washing before attempting to use either of these stains.

BLOOD CULTURES

Blood culture is indicated in any fever of obscure origin. It is the most reliable method of diagnosis in typhoid and paratyphoid fevers and in acute endocarditis, and should be done whenever the temperature remains elevated for several days without apparent cause.

Obtaining Blood for Cultures.—A Luer all-glass syringe of at least 10 c.c. capacity is sterilized by boiling. To insure absolute sterility, the boiling is continued for one-half hour to destroy spores. The blood is usually obtained from the median basilic vein at the elbow. If, for any reason, the arm veins are not available, the internal saphenous vein as it passes over the internal malleolus may be used. The skin is rendered aseptic by painting with tincture of iodin; the excess of the iodin is then removed with 95 per cent alcohol. A tourniquet (gauze bandage or piece of rubber tubing) is now applied to the arm above the elbow; it should be tight enough to obstruct the circulation in the veins but not that in the arteries. If the veins do not stand out prominently, have the patient clench his fist several times. The needle should be inserted parallel to the vein and in the direction of the blood flow. Slight suction on the plunger will show when the needle is in the vein. The operator should avoid touching the needle or the skin of the area selected for puncture, lest contamination of the culture should occur. After the syringe is filled with blood, remove the tourniquet *before* withdrawing the needle from the vein to avoid producing a hematoma.

As soon as it is withdrawn from the vein, the blood should be transferred to media. For routine work, the media employed are glucose "hormone" agar and glucose meat-infusion broth (Zinsser). If it is

necessary to transport the blood some distance, it may be placed in test tubes containing sterile 2 per cent ammonium oxalate solution to prevent clotting. It is preferable, however, to make the cultures at the bedside. In cases of suspected typhoid fever, bile media should be used.

Sources of Error.—The chief cause of error in blood cultures is secondary infection of the medium through faulty technic. This may be due to (1) insufficient sterilization of the skin; (2) incomplete sterilization of syringe and needle or contamination in fitting needle to syringe; (3) slips of technic in puncturing the skin, the more important of which are (*a*) puncturing in several places, (*b*) guiding the needle with the end of the finger, and (*c*) passing the needle through the orifice of a skin gland; (4) faulty sterilization of the medium or contamination of it during inoculation.

Blood Culture in Typhoid and Paratyphoid Fevers.—As a rule, the earlier in the course of the disease the culture is made, the better the chance of recovering the organisms from the blood. In the first ten or twelve days of the disease, blood culture is positive in about 85 per cent of cases. Repeated cultures should be made, if necessary, when the case is clinically one of typhoid. After the first two weeks, the septicemia may recur, but it is apt to be transient and blood culture gives inconstant results. When there is a *relapse,* a positive culture may again be obtained. Bile media give the best results. In an analysis of 1,137 cases of typhoid fever, Coleman and Buxton [85] report the frequency of positive blood cultures at various stages of the disease as follows:

Of 224 cases during first week, 89 per cent were positive.
Of 484 cases during second week, 73 per cent were positive.
Of 268 cases during third week, 60 per cent were positive.
Of 103 cases during fourth week, 38 per cent were positive.
Of 58 cases after fourth week, 26 per cent were positive.

Pneumonia.—Pneumococcus septicemia is common in the course of lobar pneumonia. Positive cultures are reported in 30 per cent of a series of 450 cases studied by Cole [86] at the Rockefeller Hospital; repeated cultures made at frequent intervals yielded positive results in 50 per cent of the cases. The presence of a positive culture indicates a very bad prognosis, according to Cole; the pneumococcus obtained should be typed, since he finds that with type II organisms the mortality

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[85] Cited by Zinsser, H. *A Textbook of Bacteriology,* 5th ed., 1922, p. 651.
[86] Cited by Zinsser, H. *A Textbook of Bacteriology,* 5th ed., 1922, p. 464.

is 73 per cent, with type III in the blood the mortality is 100 per cent, with type IV, 52 per cent, while with type I the mortality is only 26 per cent, due, he believes, to the beneficial effects of serum treatment in type I infections. The experience in the Medical Clinic of the Cincinnati General Hospital, though based on a much smaller material than Cole's, is quite similar, in that the death rate in cases of pneumonia with pneumococcic septicemia is much higher than in those with sterile blood cultures.

Meningitis, Acute Cerebrospinal.—In meningitis, the meningococcus may often be recovered from the blood before there are definite symptoms of meningitis. In a study of 208 cases, Herrick [87] was able to demonstrate a meningococcus septicemia in 50 per cent of cases in the premeningitic stage. In such cases treated with serum intravenously as well as intraspinously, the mortality was 16 per cent as compared with 31 per cent in the patients receiving the serum intraspinously alone. The details of the methods employed are reported by Baeslack [88] and his associates.

Malignant Endocarditis.—In malignant endocarditis blood culture is positive in the great majority of cases, and is a very important aid in diagnosis. Various organisms may be encountered. A septicemia is less frequent in simple acute endocarditis. The organisms most frequently found are streptococci (especially *Streptococcus viridans*), staphylococci, pneumococci, and gonococci. The meningococcus is occasionally encountered; typhoid bacilli, colon bacilli and diphtheria bacilli have also been reported.

Septicemia.—When there is no recognizable local lesion, a septicemia may be unsuspected, and will certainly not be diagnosed with certainty, unless blood culture is performed. Streptococci are most frequently met with; staphylococci, pneumococci, *B. influenzae, B. proteus, B. pyocyaneus* are occasionally found.

Other Conditions.—As already stated, blood cultures are indicated in any fever of obscure etiology, lasting more than three or four days. The importance of *repeated cultures* cannot be over-emphasized. In *influenza,* especially when complicated with pneumonia, in *acute rheu-*

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[87] Herrick, W. W. "The intravenous serum treatment of epidemic cerebrospinal meningitis." *Arch. Int. Med.,* 1918, XXI, 541.

[88] Baeslack, F.W., Bunce, A. H., Brunelle, G. C., Fleming, J. S., Klugh, G. F., McLean, E. H., Salomon, A. V. "Cultivation of the Meningococcus intracellularis from the blood." *Jour. A. M. A.,* 1918, LXX, 684.

matic fever, in *Malta fever,* in septicemic *plague,* blood cultures may frequently be decisive in diagnosis as well as in prognosis.

SERUM AGGLUTINATION

The property of immune sera to agglutinate bacteria, first carefully studied by Gruber and Durham, was applied to the diagnosis of disease by Widal [89] in his studies of typhoid fever. Its chief clinical value has been in the diagnosis of typhoid and paratyphoid fevers.

The Widal Test.—1. *Obtaining the Blood for Examination.*—About 1 c.c. of blood is collected in a glass tube, the ends of which have been drawn out in the form of a capillary (Fig. 89). The ear or the finger is punctured to obtain the blood, which flows into the tube by capillary attraction; or the blood may be obtained from an arm vein by means of a syringe, in which case it should be transferred to a small sterile test tube. If it is necessary to send the specimen to an outside laboratory, the ends of the tube should be sealed in the flame. The *serum* is obtained

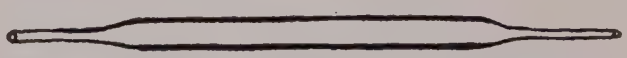

FIG. 89.—TUBE FOR COLLECTING BLOOD FOR WIDAL TEST

by allowing the blood to clot, by centrifugation, or by a combination of the two methods.

2. *Dilution of the Serum.*—The blood counting pipette may be used for making the dilution, and a white porcelain plate, such as painters use, is convenient in this connection, the different dilutions being placed in separate hollows or depressions in the plate. A 1:10 dilution of serum with normal salt solution is first prepared. From this, higher dilutions are readily made. Thus, one part of normal salt solution + one part of 1:10 dilution gives a dilution of 1:20; two parts of salt solution and one part of 1:10 dilution gives a 1:30 dilution. As the diluted serum is mixed with an equal quantity of bacterial suspension in performing the test, it follows that the resulting dilution of serum is again *doubled.* Thus, one part of 1:20 dilution + one part of bacterial suspension gives a final serum dilution of 1:40.

3. *Bacterial Suspension.*[90]—A salt solution suspension of bacilli, made

[89] Widal, F. "Sero-diagnostic de la fièvre typhoïde." *Bull. et Mém. de la Soc. méd. des Hôp.,* Paris, 1896, XIII, 561.

[90] Suspensions of killed bacilli are on the market.

from a fresh culture of typhoid bacilli, is prepared. For the private laboratory of the physician, a *suspension of the bacilli, killed by formalin,* is more convenient, as it obviates the necessity of keeping cultures on hand; furthermore, there is no danger in working with the killed bacilli. A disadvantage consists in the fact that loss of motility—one of the striking features of the reaction when living bacilli are used—cannot be observed, though agglutination is the same with living or killed bacilli.

4. *Final Dilution.*—Upon a cover glass, rimmed with vaselin, a loopful of diluted serum and a loopful of bacterial suspension are mixed, as in the usual preparation of a hanging drop, and the cover glass is then inverted over a hollow glass slide, on which the dilution is marked

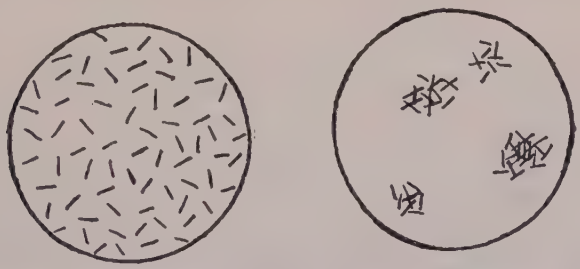

FIG. 90.—SERUM AGGLUTINATION (WIDAL TEST). Microscopic. Typical fields before and after agglutination.

with a wax pencil. Dilutions of 1:20, 1:40, 1:60, 1:80 and 1:100 are prepared.

Positive Reaction.—The bacilli are gradually agglutinated, with loss of motility, if the reaction is positive (Fig. 90). A positive reaction consists, then, in agglutination in dilutions of 1:40, 1:60 or higher *within one hour. The test should be carried out with paratyphoid bacilli A and B in addition to typhoid bacilli.*

Time of Appearance of the Widal Reaction.—The reaction rarely appears before the beginning of the second week of the disease. It may persist throughout convalescence, and often persists several months after recovery. With a chronic infection of the gall-bladder with typhoid bacilli ("carrier"), a positive agglutinin test has been found several years after the attack of typhoid fever.

Sources of Error.—A positive Widal reaction has been found in certain cases of *jaundice* and of *tuberculosis,* conditions which can usually

be excluded by clinical examination. *Vaccination for typhoid and paratyphoid fevers* is a complicating factor, since specific agglutinins may persist six months or longer following vaccination. A history of vaccination is, therefore, important in interpreting the Widal test. Though an attack of typhoid fever usually confers a lifelong immunity, instances of second attacks are encountered rarely; the agglutinins may persist in the blood from a previous attack.

SERUM AGGLUTINATION IN OTHER DISEASES

Serum agglutination has been employed in connection with other diseases, but with less success than in typhoid and paratyphoid fevers. Thus, it has been applied to the diagnosis of *bacillary dysentery* and of *Malta fever*. In experimental bacteriological studies, it has proved valuable as a means of identifying organisms, and is used for the final identification of bacilli obtained from the blood in cases of suspected typhoid fever, for example.

FIXATION OF COMPLEMENT

The Wassermann reaction is an adaptation of a fundamental observation of Bordet and Gengou [91] which has come to be known as "fixation of complement." It is known that hemolytic serum (hemolytic amboceptor, substance sensibilisatrice) is without effect upon red blood corpuscles in the absence of complement. The reaction depends upon the fact that when an antigen (that is, a substance capable of stimulating the formation of antibodies) is mixed with its inactivated immune serum (that is, immune serum heated to destroy the complement) in the presence of complement, the complement is fixed by the combined immune antibody and antigen and can no longer be found free in the mixture. If such a mixture is allowed to stand at body temperature for an hour, and to it is then added a suspension of washed red blood cells in physiological salt solution together with inactivated hemolytic serum, no hemolysis will take place, since there is no free complement to complete the hemolytic system. If, on the other hand, the original mixture contains no antibody for the antigen used, the complement present is not fixed and is available for the activation of the hemolytic serum later

[91] Bordet, J., and Gengou, O. "Sur l'existence de substances sensibilisatrices dans la plupart des serums antimicrobiens." *Ann. de l'Inst. Pasteur*, 1901, XV, 289.

added. *"In other words, the hemolytic system (that is, cells plus amboceptor) simply serves as an indicator."* [92]

The reaction thus depends upon the fact that neither antigen alone, nor amboceptor (antibody) alone can fix complement, but that this fixation is carried out only by the combination of antigen plus amboceptor. Any specific can be determined by this method, provided the homologous antigen is used; and *vice versa,* by the use of a known antibody, a suspected antigen may be determined.[93]

The Wassermann Reaction.—The Wassermann reaction is, as has already been stated, merely an adaptation of the observation of Bordet and Gengou, and it is not without reason that the French designate the test as the Bordet-Wassermann reaction. The technic is far too time-consuming and the reaction beset with too many difficulties to make it a test which the practicing physician can employ in his private laboratory, without the assistance of a trained technician. It is a test which, however, is so universally employed that it is essential that all physicians understand the underlying principles of it. The original antigen used in the Wassermann reaction was prepared by the extraction of syphilitic livers. It has since been shown that the reaction is not specific and that suitable antigens may be prepared by alcoholic extraction of many normal organs of man and animals.

The following description of the reaction is given by Rosenau: [94] Two antigens are used; one is an alcoholic extract prepared from human heart muscle and half saturated with cholesterin at 17° C., the other is prepared in like manner from guinea pig hearts. Each of these antigens is diluted with 0.85 per cent salt solution before testing, in the proportion of 4 parts of the antigen extract to 16 parts of the salt solution. The amount to be used—the dosage—is carefully determined by testing each antigen against a large number of known positive and known negative specimens of blood. Any antigen which gives a positive reaction with a known negative specimen of blood ("false positive") is unsuitable for testing and should be rejected. Further, it is unsafe to employ an antigen when twice its dosage inhibits hemolysis when incubated for one hour with "the hemolytic system" consisting of complement and the hemolytic mixture (sensitized red blood cells). Usually

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[92] Walker, Robert. Personal communication.
[93] Zinsser, H. *A Textbook of Bacteriology* (5th ed.), N. Y., 1922, 315.
[94] Rosenau, M. J. *Preventive Med. and Hygiene* (4th ed.), N. Y., 1921, 581.

0.1 c.c. to 0.2 c.c. of the diluted antigen is used. Lecithin and other lipoidal substances may also act as antigen.

Syphilitic antibody in the patient's serum to be tested is the unknown quantity. This serum is heated in a water bath at 55° C. for 30 minutes to destroy its complement. One-tenth c.c. of the patient's serum is used in *the test* and 0.2 c.c. is used for *the serum control.* The serum control indicates the presence or absence of inhibiting (anticomplementary) substance other than specific antibodies.

The *complement* is contained in the serum of freshly drawn guinea pig's blood which has been kept at 37° C. for one to two hours. A 10 per cent solution of this serum in physiological salt solution constitutes the complement. The amount used in the test is twice the minimum necessary to hemolyze a definite quantity of sensitized cells. Usually this is from 0.4 c.c. to 0.5 c.c. of the complement.

Washed corpuscles are prepared from freshly obtained, defibrinated sheep's blood. In order to free the corpuscles of serum, the blood is washed three times with from four to five volumes of physiological salt solution at each washing. Finally, the volume of cells and salt solution is made equal to the volume of defibrinated blood originally used. Such a suspension is called washed sheep's corpuscles, from which the 5 per cent suspension for the test is prepared.

Immune rabbit's serum is prepared by injecting washed sheep's corpuscles into the peritoneal cavity of a rabbit at three day intervals, namely, first injection 7 c.c.; second, 14 c.c.; third, 21 c.c.; and finally, 28 c.c. The rabbit is bled on the 9th or 10th day after the last injection, and the clear serum obtained from its blood is heated in a water bath at 55° C. for one-half hour to destroy complement. This heated rabbit's serum contains the hemolytic amboceptor and is diluted with 0.85 per cent salt solution, so that 0.25 c.c. will hemolyze 0.5 c.c. of a 5 per cent suspension of sheep's corpuscles. In the test 0.5 c.c. of the diluted amboceptor is used. A small quantity of the rabbit's serum is freshly diluted for each day's test.

The *hemolytic mixture, or sensitized cells,* consists of equal parts of a 5 per cent suspension of washed sheep's corpuscles and the diluted amboceptor which have been incubated together in a water bath at 37° C. for one-half hour to completely sensitize the red corpuscles. One c.c. of sensitized cells is used in the test.

The Test.—Mix the patient's serum (Fig. 91) or spinal fluid with the antigen and the complement, according to the table on page 362. The

tubes are well shaken and the whole is incubated at 37° C. for 40 minutes.[95] At the end of this time all of the complement has been fixed in those tubes which contain syphilitic antibody, antigen, and complement. Finally, 1 c.c. of sensitized cells is added to each tube, and the whole again incubated at 37° C. for one hour. The results of the Wassermann test are then read.

The *absence of hemolysis* indicates the *presence* of the syphilitic antibody in the patient's serum, and therefore a *positive* reaction. The *presence of hemolysis* indicates the *absence* of the syphilitic antibody in the patient's serum, and therefore a *negative* reaction. *Partial hemolysis* signifies a *doubtful* reaction. It is advisable to test several

Fig. 91.—Keidel Tube for Collecting Blood from the Vein.

specimens from such a case, and to interpret a persistently or predominatingly doubtful reaction as indicative of a syphilitic infection.

Occurrence.—A positive Wassermann test in the blood is obtained in from 80 to 95 per cent of all cases of syphilis with clinical manifestations. It appears from the end of the second to the end of the fourth week, becomes more marked, and may continue for an indefinite period. During active treatment it may be absent, to reappear again. Its intensity bears some relation to the activity of the lesions (Osler). The reaction is positive in the spinal fluid in practically all cases of general paresis and in the great majority of the cases of tabes dorsalis. It is in latent cases of syphilis that the Wassermann test is least dependable, about 50 per cent of the cases giving a negative reaction.

Sources of Error.—In the tropics, *yaws or framboesia,* a disease in some respects similar to syphilis and caused by the *Treponema pertenue,*

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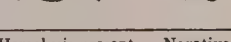
[95] Instead of fixing the complement by incubation, the tubes may be placed in the ice box for 10 hours at a temperature not exceeding 2° C. This modification, proposed by Jacobstahl, has been investigated by McNeil, Ruediger and others. A recent review is that of McIntyre, H. D., North, E. A. and McIntyre, A. P. (''Comparative values of complement fixation methods in syphilis.'' *J. Lab. and Clin. Med.*, 1921, VI, 233), who find that the ice box Wassermann reaction is the first to appear positive; it is also, they say, the last to become negative as a result of treatment.

WASSERMANN TEST

	Number of tube	*Antigen* Alcoholic extract of human heart cholesterinized *	*Antibody* *Patient's Serum* heated 55°C for 30 minutes **	*Complement* Guinea-pig serum 10% †	*Saline Solution* 0.85%	Incubate 40 minutes in water bath at 37° C.	*Hemolytic Mixture* 5% suspension of sheep's corpuscles + heated rabbit's immune serum (amboceptor) §	Incubate for 1 hour in water bath at 37° C. Note results immediately and again after having kept in ice box for 12 to 14 hours.	Result
Antigen Control	I	0.2 c. c.	0.0 c. c.	0.5 c. c.	0.8 c. c.		1.0 c. c.		Hemolysis should be complete
Serum Control	II	0.0	0.2	0.5	0.8		1.0		Hemolysis should be complete
Serum Test	III	0.1	0.1	0.5	0.8		1.0		No Hemolysis = syphilitic antibody in patient's serum (Positive) Hemolysis = absence of syphilitic antibody in patient's serum (Negative) Partial Hemolysis = Doubtful result
Diagrammatic Representation of Positive and Negative Test		Antigen	Syphilitic Antibody	Complement			Hemolytic Mixture		Hemolysis absent = Positive
			Syphilitic Antibody (Absent)						Hemolysis present = Negative 

For routine tests the positive serum of a known syphilitic, the negative serum of a non-syphilitic and each of the serums (or spinal fluids) to be tested are set up according to tubes No. II and No. III, together with one antigen control according to tube No. I.

* The dilution is made so that 0.1 c. c. contains one-half the maximum amount or less than one-half the maximum amount which does not inhibit hemolysis. A carefully standardized alcoholic extract of human heart muscle, half-saturated with cholesterin, usually gives a superior antigen. (See text.)

** Use five times the quantity of spinal fluid not heated.

† Five-tenths cubic centimeter contains two units, as determined against the hemolytic mixture.

§ The amount of immune rabbit's serum in each c. c. of this mixture is twice that necessary to hemolyze 0.5 c. c. of a 5% suspension of sheep's cells in the presence of 0.5 c. c. of 10% complement.

may give a positive reaction; likewise, *tubercular leprosy* at times gives a positive test. It is occasionally found to be positive, too, in *malaria,* and in *relapsing fever.*

COMPLEMENT FIXATION IN GONOCOCCAL INFECTIONS

In gonorrhea and its complications, complement fixation has proved to be of great assistance in diagnosis. A polyvalent antigen, such as McNiel recommends, is used. It is prepared from cultures of the ten strains of gonococci separated by Torrey. The reaction is specific.

The results of the complement fixation reaction vary with the localization and duration of the infection.[96] Thus, a negative reaction frequently occurs in the presence of disease, especially in the acute or subacute stages when the gonorrhea is limited to the urethra; and it is always negative when the disease is confined to the anterior urethra or vagina. In their study of 204 cases, Thomas and Ivy [96] found the reaction positive in 21 per cent of patients *clinically cured.* The test is often valuable in detecting a previous infection with the gonococcus. In only 9 per cent of cases of *acute and subacute anteroposterior urethritis* was the reaction positive; and the earliest positive test was obtained in the sixth week of the disease. On the other hand, in a number of cases of *chronic recurrent urethritis with acute exacerbation,* the test was invariably positive; many no doubt had prostatitis. In *chronic posterior urethritis,* a positive test was found in 33 per cent of cases; in *chronic prostatitis* in 52 per cent; in *stricture* of the urethra in 66 per cent. With *epididymitis* complement fixation was positive in 87 per cent, while in cases of *arthritis,* undoubtedly gonococcic in origin, positive tests were obtained in 100 per cent of cases. In *pelvic inflammations in women,* the test is an important aid in differential diagnosis;[97] it appears that the infection must ascend at least to the level of the uterus in order to produce a positive blood reaction. It should be recalled that treatment with *gonococcic vaccine or immune serum* may lead to a positive test.

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[96] Thomas, B. A. and Ivy, R. H. (*a*) "The gonococcus complement-fixation test and analysis of results from its use." *Arch. Int. Med.,* 1914, XIII, 143. (*b*) *Applied Immunology* (2d ed.), Phila., 1916, 146 *et seq.*

[97] Smith, J. D. and Wilson, M. A. "Comparison of smear, culture and complement fixation in chronic gonorrhoea in women." *Jour. Immunol.,* 1920, V, 499.

COMPLEMENT FIXATION IN TUBERCULOSIS

The results of complement fixation in tuberculosis are promising, though it is not of such value at present as in syphilis or in some of the complications of gonorrhea. The reaction is not specific. Rogers [98] has found that, after the tubercle bacilli, other acid-fast bacilli, such as *B. smegmatis, B. leprae* and Moeller's *grass bacillus* gave the higher degree of fixation. Other substances, he found, such as *staphylococci, B. coli, B. subtilis* and concentrated solution of *peptone* gave occasional fixations. Rieux and Bass [99] have likewise obtained positive reactions in some cases of *syphilis* and of *malaria* (with parasites in the blood).

In *pulmonary tuberculosis* it has been found that the reaction is generally negative in very early cases and in those rapidly advancing. Craig [100] found a positive reaction in 96 per cent of cases of active tuberculosis and in 66 per cent of clinically inactive tuberculosis. Rieux and Bass found 77 of 78 cases of pulmonary tuberculosis with positive reactions; the negative was in a patient dying of tuberculous pneumonia. They also report 51 of 80 cases of clinically latent tuberculosis positive with the complement fixation test. Von Wedel [101] has reported the results of 6,128 complement fixation tests made on 1,207 sera from 1,000 patients with tuberculosis. He, too, found that many patients with incipient and with far advanced disease give a negative reaction. From his own experience and an analysis of the literature, he concludes that about 70 per cent of cases give a positive complement fixation reaction. In the majority of cases he finds the positive test confirmatory but not essential for the diagnosis. A *positive* reaction *repeated twice* seems to prove fairly conclusively that the patient has an active tuberculous process. He further concludes that the weakening of the reaction from a strongly positive to a weakly positive or negative reaction is a favorable prognostic sign in incipient or moderately advanced cases exhibiting

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[98] Rogers, J. B. "Complement fixation in tuberculosis and a comparison of the Wassermann and Hecht-Weinberg-Gradwohl systems." *Jour. Infect. Dis.*, 1920, XXVII, 101.

[99] Rieux, J. and Bass. "Réaction de fixation (antigène de Besredka) et tuberculose." *Ann. de l'Inst. Pasteur*, 1921, XXXV, 378.

[100] Craig, C. F. "Observations upon complement fixation in the diagnosis of pulmonary tuberculosis." *Am. Jour. Med. Sc.*, 1915, CL, 781.

[101] Von Wedel, H. O. "The complement fixation test for tuberculosis." *Jour. Immunol.*, 1920, V, 159-225. (Literature.)

clinical improvement, while the same change in the reaction in far advanced cases in poor clinical condition indicates a bad prognosis.

In 16 of 28 cases of *serofibrinous pleurisy,* the test was positive; likewise, in 4 of 6 cases of *tuberculous peritonitis.*

Fried and Moser [102] studied 869 cases of *bone, joint* and *lymphatic gland diseases,* of which 713 were tuberculous; the ages of the patients varied between 2 and 14 years. They find that, with few exceptions, a positive reaction indicates the existence of an active tuberculous focus; a negative reaction, on the other hand, does not enable one to exclude tuberculosis. The proportion of positive reactions, high in the initial stage of bone and joint tuberculosis, falls when the lesions cicatrize. They also obtained a positive reaction in 10 per cent of 100 cases of rickets and in 6 per cent of 56 cases of nontuberculous bone disease.

From the foregoing brief review, it is apparent that, while assistance may be obtained from the reaction in the study of tuberculosis, complement fixation is not specific, and its interpretation, when positive, is often difficult.

COMPLEMENT FIXATION IN OTHER DISEASES

Complement fixation has been used in other diseases than those noted in the preceding pages, but the results thus far have not been such as to lead to the general adoption of the method.

Typhoid Fever.—In using complement fixation in typhoid fever, a highly polyvalent antigen is essential. Garbat [103] has studied the reaction and finds that the test is occasionally positive before the blood culture or Widal, but usually not before the end of the second week. Generally, the reaction persists for six weeks after recovery.

Influenza.—In influenza, Cooke [104] has found complement fixation in a considerable number of older children and adults during convalescence. The results are of interest in showing the apparent relation of the bacilli to the disease, but are of little practical diagnostic value.

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[102] Fried, B. and Moser, M. "Réaction de fixation à l'antigène de Besredka dans la tuberculose externe." *Ann. de l'Inst. Pasteur,* 1921, XXXV, 388.

[103] Garbat, A. L. "The complement fixation test in typhoid fever: Its comparison with the agglutination test and blood culture method." *Am. Jour. Med. Sc.,* 1914, CXLVIII, 84.

[104] Cooke, J. V. "Complement fixation in influenza with B. influenzae antigens." *Jour. Infect. Dis.,* 1920, XXVII, 476.

Whooping Cough.—Using an antigen prepared from cultures of the *B. pertussis* of Bordet and Gengou, Winholt [105] has found that complement fixation occurs about two weeks after the onset of the disease. Fixation is not as strong at this time as it is eight to ten weeks after the onset of the disease. The reaction is specific. Tests of the sera with influenza antigen were negative.

DISEASE CARRIERS [106]

By the term "carrier," one designates a person in good health who harbors pathogenic bacteria, protozoa or animal parasites. Carriers are important factors in the spread of disease. The more important carriers and the means by which they are detected follow.

Disease	*Means of Detecting Carrier*
Typhoid and paratyphoid fevers	Cultures of urine and feces
Influenza	Cultures of sputum and of throat
Acute cerebrospinal meningitis	Cultures of nasopharynx
Diphtheria	Cultures of tonsils, pharynx, nose
Bacillary dysentery	Cultures of feces
Cholera	Cultures of feces
Pneumonia	Cultures of throat
Gonorrhea	Stained smears and complement fixation
Amebic dysentery	Cysts of ameba in feces
Malaria	Sexual forms of parasite in blood; splenic puncture
Syphilis	Complement fixation; spirochetes
Leishmaniasis	Parasites from splenic or hepatic puncture
Tuberculosis	Examination of sputum
Animal parasites	See table, p. ?
Skin parasites	See Chapter VII

[105] Winholt, W. "Complement fixation test in whooping cough." *Jour. Infect. Dis.*, 1915, XVI, 389.

[106] A good discussion of this problem is that of C. E. Simon, *Human Infection Carriers.* Phila. and N. Y., 1919, and of H. J. Nichols, *Carriers in Infectious Diseases,* Balto., 1922.

CHEMISTRY OF THE BLOOD

By Raphael Isaacs, A. M., M. D.,

and

David S. Hachen, B. S., M. D.

In recent years, the chemical analysis of the blood has assumed a position of prominence in clinical medicine. The subject is of particular interest because the advance in this field is due almost entirely to work in American laboratories. The results of chemical analyses of the blood are of value in diagnosis, prognosis, and control of treatment, especially in such conditions as renal insufficiency, diabetes mellitus, endocrine disturbances, and gout, and in the differential diagnosis of such conditions as uremia, diabetic coma, cerebral hemorrhage, acute intoxications; diabetes mellitus and renal diabetes; the functional stages of endocrine disorders. New methods are constantly being devised, their value in various diseases is being more widely studied, and with simplification of technic their application is becoming more extended. Like all other laboratory methods, they cannot replace a full history and physical examination, but are often invaluable in completing the study of a patient. In coma, for example, the chemical examination of the blood may be the decisive factor in the differential diagnosis. In the table on page 368, as well as in the following pages, the diseases in which the methods have been found most useful are enumerated.[107]

Additional Data on the Chemistry of the Blood in Disease.[108]

Acute alcoholism. Carbon dioxide combining power of plasma 40 to 60.

Addison's disease.

Creatinin, uric acid, carbon dioxide combining power and sugar (60 to 90) decreased.

Albuminuria, Orthostatic. Nonprotein nitrogen, uric acid, urea N, creatinin, within normal limits.

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[107] For the special worker in blood chemistry, the volumes of O. Folin, *A Laboratory Manual of Biological Chemistry*, N. Y., 1922, and of V. C. Myers, *Practical Chemical Analysis of Blood*, St. Louis, 1921, are of great value, as well as frequent papers, appearing chiefly in the *Journal of Biological Chemistry.*

[108] Observations on only one or two constituents.

NORMAL AND PATHOLOGICAL BLOOD CHEMICAL ANALYSES

Figures in Mg. per 100 c.c. whole Blood. (After 12–14 hours fast.)	Non-protein Nitrogen	Urea Nitrogen	Creatinin	Uric Acid	Sugar	CO_2 Combining Power of Plasma	Chlorides as NaCl (Whole Blood)	Cholesterol	Fat
Normal	25–40	12–20	1–2	2.0–4.0	60–120	53–77	450–520	140–170	600–700
Bichloride Nephritis	+ to 370	90–300	10–33	8–15	120–200	25–36	114–410	340–350	..
Diabetes Insipidus	N.	N. or −	N.	N.	N.	N.	N.		
Diabetes Mellitus	N.	N.	N.	N.	105–1200	13–60	N.	150–300	300–1100
Eclampsia	N.	10–25	N. to +	N. to 11.7	N. or +	43–58	N. or +	+ to 230	
Eczema	N.	9–26	− to +	N. to +	N. to 180	28–62		N. to 197	
Erythremia	+	N.		N. to 8.0	N.	N. or −			+
Furunculosis	N.	N.	N.		N. to +	− or N.			
Gout	N.	N. to 35	N.	N. to 10	N. to 140	N.			
Hyperthyroidism		N.	N.		N. to 157	N. or −			
Hypertension (Essential)	N.	N. to 29.9	N. to 2.3	N. to 6.95					

Hypertension (with early Nephritis)	28–56	11–32	1.5–2.2	2.5–9.0					
Leukemia	N.	N.	N.	N. to 5.3	N.		N. or +	–	+(serum)
Malignancy (Advanced)	N. or +	+	N.	+ to 18.1	– to 41	–	+	–	
Myocardial Insufficiency	N.	N. or +	N. or +	N. or +	N. or +	– or N.			
Pernicious Anemia	N. to +	N.	N.	N. +or –	N. or +		+ to 600	600–700	
Pneumonia					N. or +	N. or –	–	–	
Polycystic Kidney (Double)	N. to 400	+ to 75	+ to 8.0	+ to 5.0	+ to 200				
Pregnancy	N.	N.	N.	N.				+	
Prostatic Obstruction	N. to +	N. to 40	N. to 3.5	N. to 9	110–160				
Psoriasis	N.	N.	– or N.		74–220	38–60			
Renal Insufficiency	40–460	20–375	2–25	+ to 18	75–160	12–75	N. – +	170–350	
Syphilis Uncomplicated	N. or +	6–32	N. to 2.4	N.	N. to 137	N.		+	
"Thermic" Fever		26–89	3–6.1	6–14	150–200				
Uremia	90–400	70–300	4–35	4–25	N. to 200	20–70	450–650	170–350	

+ = Increased. – = Decreased. N = Within normal limits.

Anemia (Secondary).

Sugar increased. Fat increased in plasma when corpuscles are below 50 per cent.

Arsenobenzol poisoning. Carbon dioxide combining power of plasma 29 to 40.

Arteriosclerosis.

Cholesterol 210 to 230.

Arthritis, rheumatoid.

Uric acid increased at times.

Beriberi.

Fat 200.

Chlorosis.

Cholesterol decreased.

Cholelithiasis.

Cholesterol 130 to 300.

Cholera.

Chlorides 352 to 431.

Cretinism.

Sugar 60 to 90.

Hemolytic jaundice.

Cholesterol decreased.

Miliary tuberculosis. Carbon dioxide combining power of plasma 52.

Muscular dystrophy.

Sugar 64 to 86.

Myxedema.

Sugar 60 to 90.

Pellagra.

Cholesterol 240.

Pulmonary tuberculosis.

Sugar increased or decreased. Carbon dioxid combining power of plasma 48 to 73.

Renal diabetes.

Urea nitrogen 16.3 to 18.9. Sugar 68 to 120.

Tetanus. Carbon dioxid combining power of plasma 39 to 55.

Xanthoma. Sugar may be increased to 130. Cholesterol to 210.

Method of Taking Blood for Chemical Examination.—Dry 5 drops of a 20 per cent solution of potassium oxalate in a test tube. Collect 15 c.c. of blood from an arm vein with a syringe, and after transferring

to the test tube, rotate rapidly to secure thorough oxalation. Blood *sugar* determination should be made at once; *all others* may be postponed for twenty-four hours, but in this case the filtrate should be made and should be preserved by the addition of a few drops of toluol. If one drop of 40 per cent formaldehyd solution [109] is added to each 5 c.c. of blood, the blood may be preserved for *sugar* or *nonprotein nitrogen* determinations, even if kept at room temperature, for three days. The specimen should be collected in the morning, before breakfast, after a twelve- to fourteen-hour fast.

In collecting blood for the determination of the *carbon dioxid combining power* of the plasma, the following precautions should be observed. The patient must be at rest for at least one hour before the blood is drawn. Venous stasis should be avoided in collecting blood, by temporarily releasing the tourniquet as soon as the needle pierces the vein. The stagnant blood will pass in a few seconds, and the specimen may then be collected and oxalated as above. In aspirating, use a minimal amount of suction and avoid free air space in the barrel of the syringe. Exposure of the blood to the air is avoided by overlaying with liquid petrolatum. The corpuscles should be centrifuged off as soon as possible and the plasma removed. If it is necessary to postpone the analysis, the plasma may be preserved with a drop of toluol, and kept on ice for a week, if necessary. "Non-soluble" test tubes are preferred.

Method of Preparing Protein-Free Filtrate.[110]—For determination of sugar, nonprotein nitrogen, urea, creatin and creatinin, uric acid and chlorids.

Reagents:

1. Sodium tungstate	11.2 gm. of	$Na_2WO_42H_2O$
Distilled water to	100.0 c.c.	
2. Sulphuric acid, concentrated c.p.	3.5 gm.	
Distilled water to	100.0 c.c.	

Method.—1. To one volume of blood (2 to 15 c.c.) add seven volumes of distilled water, in a 250 c.c. Erlenmeyer flask, shake and set aside until fluid is transparent (complete laking).

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[109] This is the strength of the commercial solution.

[110] Folin, O. and Wu, H. "A System of Blood Analysis." *Jour. Biol. Chem.*, 1919, XXXVIII, 81.

2. Add one volume of sodium tungstate solution.

3. Add one volume of the sulphuric acid solution, drop by drop, shaking.

4. After thorough mixing, allow to stand for 5 minutes. The mixture should be practically bubble-free, and should turn from bright red to dark brown in color.

5. Filter, wetting the filter paper first with a few c.c., before adding

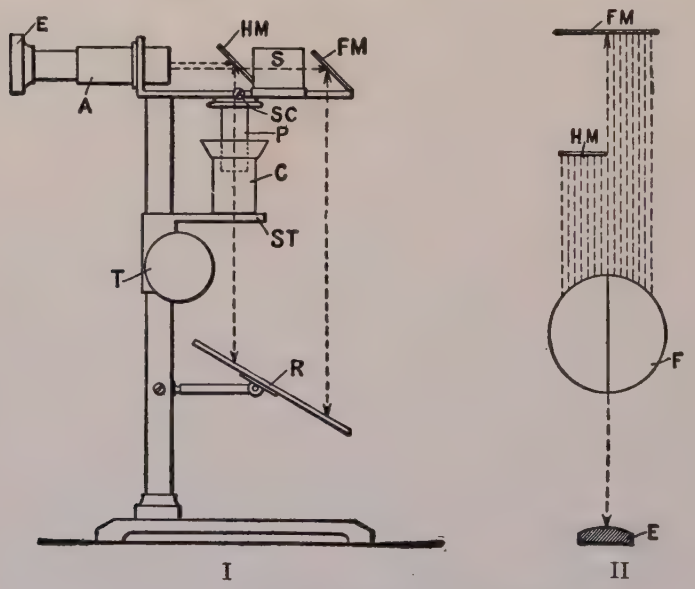

Fig. 92.—The Bock-Benedict Colorimeter.

I. *A*, Lens; *E*, Eye-piece; *HM*, Half size mirror; *FM*, Full size mirror; *S*, Cell for standard solution; *P*, Plunger; *C*, Cup for unknown solution; *R*, Large reflector; *ST*, Stage for holding cup; *T*, Thumb screw moving; stage up or down; *SC*, Set screw.

II. *FM*, Full size mirror; *HM*, Half size mirror; *F*, Side by side field; *E*, Eyepiece.

the bulk of the mixture. The filtrate should be water-clear, and nearly neutral to Congo-red (10 c.c. of filtrate requiring not more than .2 c.c. of $\frac{N}{10}$ sodium hydroxid when titrated, using phenolphthalein as the indicator). In case the determination is not to be made at once, a few drops of toluol or xylol should be added to the filtrate, for preservation, and the flask stoppered.

Directions for the Use of Colorimeter (*Duboscq and Bock-Benedict Types*).—Place the cups in position and adjust the mirrors, so that

both fields seen through the eyepiece have the same illumination. Samples of the same solution in both cups (Duboscq type) or in the cup and the cell (Bock-Benedict type, Fig. 92) should give the same readings. If not, the degree of error must be noted, and the readings corrected accordingly.

For use, the unknown solution is placed in one cup and the standard solution in the other cup or in the rectangular cell, so that the plunger dips into the cup. The cell of the Bock-Benedict instrument measures 15 mm. in one direction and 20 mm. in the other direction. The standard is usually set at 20. The cup containing the unknown is slowly moved up and down, and the colors compared through the eyepiece. All readings must be made with the cover (metal housing) in place. The average of 5 readings is taken. The reading of the unknown is comparable in inverse ratio to the standard.

The general formula for blood is,

$$\frac{S}{R} \times \text{strength of standard (in mg.)} \times \frac{100}{\text{No. of c.c. of blood represented in unknown solution.}} =$$

number of milligrams of unknown in 100 c.c. of blood.

S = Reading of standard (mm.) on colorimeter.

R = Reading of unknown (mm.) on colorimeter.

BLOOD SUGAR [111]

Significance.—The *normal* blood sugar is from 0.08 to 0.12 per cent. After a meal rich in carbohydrate, there may be an appreciable rise in the sugar content of the blood (hyperglycemia) even to such an extent that sugar may pour over into the urine (glycuresis). The normal blood threshold of sugar excretion (that is, the point of glycuresis) is about 0.16 to 0.18 per cent. In *renal diabetes,* the threshold point is lower than normal, while in cases of *nephritis* the threshold point is higher than normal. Thus, in cases of diabetes complicated by nephritis,

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[111] Folin, O. and Wu, H. "A Simplified and Improved Method for Determination of Sugar." *Jour. Biol. Chem.*, 1920, XLI, 367.

the blood sugar may go above 0.3 per cent before sugar appears in the urine.

In mild cases of *diabetes mellitus,* the blood sugar varies between 0.2 and 0.3 per cent and in severe cases figures up to and even above 1.0 per cent have been obtained.

Hyperglycemia is present in *hyperthyroidism, nephritis* and *malignancy.* In the last two cases the high blood sugar is a retention phenomenon. Hypoglycemia (around 0.06 per cent or below) has been reported in *myxedema, cretinism, Addison's disease* and *muscular dystrophy.*

Reagents:

1. *Molybdate Phosphate Solution.*

Molybdic acid	35.0 gm.
Sodium tungstate	5.0 gm.
Sodium hydroxid (10 per cent)	200.0 c.c.
Distilled water	200.0 c.c.

Boil off any ammonia present for 20 to 40 minutes. Cool, dilute to 350 c.c. Add 125 c.c. of conc. (85 per cent) phosphoric acid. Dilute to 500 c.c.

2. *Alkaline Copper Solution.*

Sodium carbonate (anhydrous)	40.0 gm.
Distilled water about	400.0 c.c.
Tartaric acid	7.5 gm.
Dissolve	
Copper sulphate (crystallized)	4.5 gm.
Distilled water to	1,000.0 c.c.

3. *Standard Sugar Solution* (Stock).[112]

Glucose (anhydrous)	1.0 gm.
Distilled water to	1,000.0 c.c.
Toluol few drops	
When ready for use:	
Stock solution	2 c.c.
Distilled water to	10.0 c.c.

[112] Modification used in Laboratory in Cincinnati General Hospital.

Method.—To a Folin blood sugar test tube (Fig. 93), add 2 c.c. of the clear, tungstic acid blood filtrate (See p. 371). To two other similar tubes add 1 c.c. and 2 c.c. of the diluted *standard* sugar solution, prepared as above. To the first standard tube, add 1 c.c. of water to equalize the volumes. To the three tubes, add 2 c.c. of alkaline copper solution. Transfer for 6 minutes to a boiling water bath. Then cool, without shaking, in a beaker of water for 2 to 3 minutes.

To each tube add 2 c.c. of molybdate phosphate solution. When the effervescence has subsided and the solution is a clear blue, dilute to the 25 c.c. mark, insert a rubber stopper and mix.

The unknown solution is then compared with the standard, which it most nearly resembles, in a colorimeter.

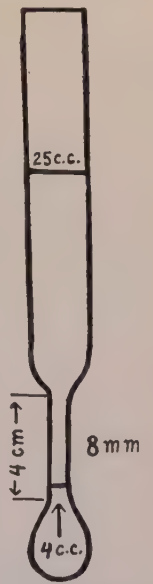

FIG. 93.—FOLIN SUGAR TUBE.

Weaker Standard:

$$\frac{\text{Reading of standard (mm.) (usually 20)}}{\text{Reading of the unknown (mm.)}} \times 100$$

= number of milligrams of glucose in 100 c.c. of blood.

Example:

Reading of standard 20
Reading of unknown 14

$$\frac{20}{14} \times 100 = 142.8 \text{ mg. of glucose in 100 c.c. of blood.}$$

Stronger Standard:

Multiply the result above by 2.

GLUCOSE TOLERANCE TEST [113]

Significance.—In a *normal adult* the difference between the blood sugar before and two hours after the administration of 1.75 gms. of glucose per kilo body weight on a fasting stomach seldom exceeds 0.01 per cent. The maximum of blood sugar concentration is 0.15 per cent and is reached approximately one hour after the ingestion of the glucose. Cases in which the maximum of blood sugar concentration is greater than 0.15 per cent and the return to normal is delayed for 3 hours or

[113] Janney, N. W. and Isaacson, V. I. "A Blood Sugar Tolerance Test," *Jour. A. M. A.*, 1918, LXX, 1131.

more, are said to have a decreased tolerance for sugar. The tolerance is highest *below three years of age,* and is lowest in *old age.* A decreased

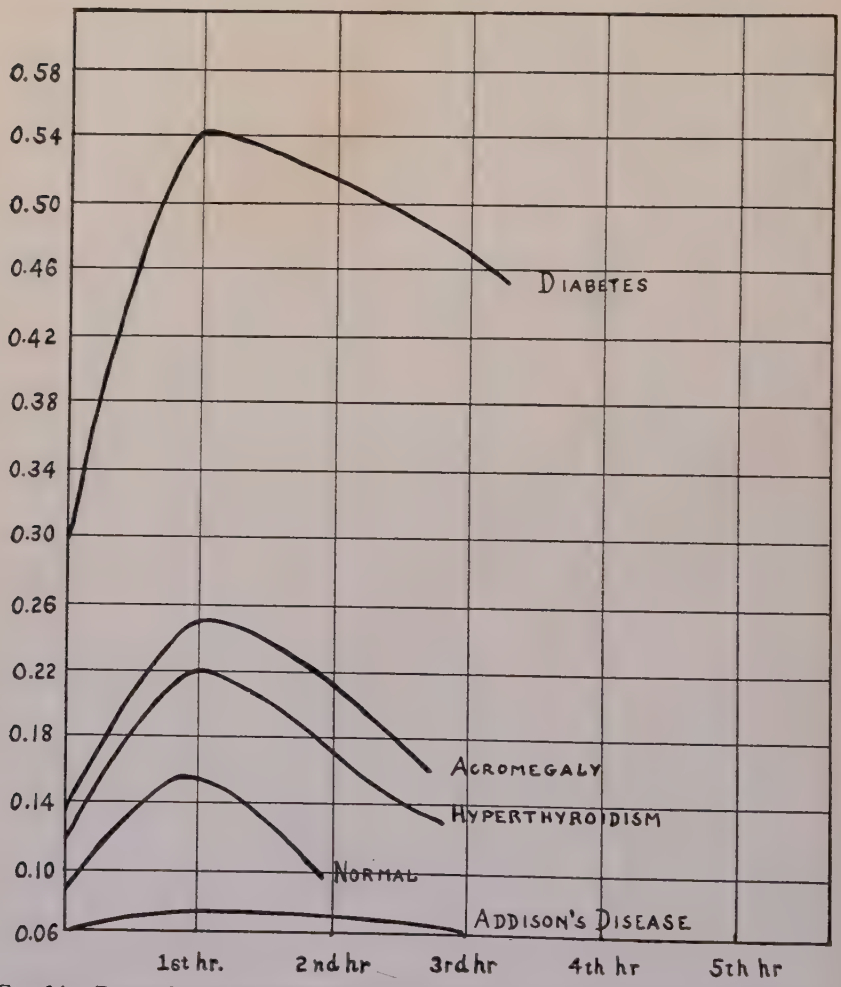

FIG. 94.—BLOOD SUGAR TOLERANCE CURVES IN DIABETES, MELLITUS, ACROMEGALY, HYPERTHYROIDISM AND ADDISON'S DISEASE, AS COMPARED WITH THE NORMAL. The vertical line represents the percentage of blood sugar.

tolerance is found in *diabetes, nephritis, cancer, hyperthyroidism* and *acromegaly.* In nephritis the high blood sugar is a retention phenomenon. The decreased tolerance in old age may be confused with that in nephritis

and cancer. In certain other endocrine disturbances, such as *cretinism, myxedema, Addison's disease* and also in *muscular dystrophy,* the blood sugar remains persistently low after glucose administration. Curves in diabetes, acromegaly, hyperthyroidism, and Addison's disease, together with the curve of a normal case, are shown in Fig. 94.

Technic of the Blood Sugar Tolerance Test.—The patient fasts from 7 P.M. one day until the test is completed the following morning. At an early morning hour the blood sugar is determined. Then 1.75 gm. of glucose per kilo of body weight is given by mouth. A 40 per cent cooled glucose solution is used, flavored with the juice of one lemon. Exactly two hours after the ingestion of the glucose, the blood sugar is again determined. If the tolerance is normal, the difference between this estimation and the fasting value should not exceed 0.01 per cent.

NONPROTEIN NITROGEN IN THE BLOOD

Significance.—*Normal* blood contains 25 to 40 mgms. of nonprotein nitrogen per 100 c.c. The nonprotein nitrogen constitutes about one per cent of the total nitrogen of the blood and includes the nitrogen present in urea, uric acid, creatinin, creatin, amino-acids and ammonia. The so-called "rest N" or undetermined nitrogen makes up about 46 per cent of the normal nonprotein nitrogen. After a meal containing protein, there is a temporary elevation in the nonprotein nitrogen of the blood. In early *interstitial nephritis,* there may be only a slight rise in the uric acid or urea, although in the terminal stages of the disease, there is a marked elevation in all the forms of nonprotein nitrogen. A fatal prognosis, with death within a few days or a week is indicated when the nonprotein nitrogen reaches .275 per cent.

Reagents:

1. *Sulphuric-Phosphoric Mixture.*

Phosphoric acid (syrup, 85 per cent)	300.0 c.c.
Sulphuric acid (conc.) (ammonia-free)	100.0 c.c.

Mix and allow sedimentation of impurities (calcium sulphate) for at least a week, excluding the absorption of ammonia, by covering tightly.

Above solution (supernatant clear fluid)	100.0 c.c.
Copper sulphate solution (6 per cent)	10.0 c.c.
Distilled water	100.0 c.c.

2. *Standard Ammonium Sulphate Solution.*

Ammonium sulphate c.p. dried	0.4716	gms.
Distilled water to	1,000.0	c.c.

3. *Nessler's Solution.*

Potassium iodid	150.0	gms.
Iodin crystals	110.0	gms.
Distilled water	100.0	c.c.
Metallic mercury	150.0	gms.

Shake the above mixture continuously for 7 to 15 minutes. The solution becomes quite hot. When the red iodin solution turns pale, cool in running water until the reddish color of the iodin has been replaced by the greenish color of the iodid, the process taking about 15 minutes. Decant, and wash the surplus mercury. Dilute the solution and washings to 2 liters, and store as a "stock double iodid" solution.

Sodium hydroxid.

(Supernatant liquid from a saturated aqueous solution, that is, 50 per cent to 55 per cent NaOH)	750.0	c.c.
Distilled water to	3,500.0	c.c.

(Determine by titration [with 2N H_2SO_4] that this is a 10 per cent solution within an error of 5 per cent.)

Sodium hydroxid (10 per cent sol.)	3,500.0	c.c.
Double iodid solution (see above)	750.0	c.c.
Distilled water to	5,000.0	c.c.

The solution will be a clear, straw color. A light brown, granular precipitate will form on standing several days, but the supernatant fluid may be used. The solution improves with age.

Method.—Use a thoroughly dry 75 c.c. ("pyrex") test tube, graduated at 35 c.c. and 50 c.c.

Protein-free blood filtrate (see p. 371)	5.0	c.c.
Sulphuric-phosphoric acid mixture	1.0	c.c.

Support the test tube in a clamp on a ring stand, add a glass bead, and boil vigorously over a small flame 3 to 7 minutes, until the steam is replaced by white fumes. If the burner is moved to one side of the tube, the bumping can be more easily controlled. The solution becomes dark in color before the fumes appear. When these fill the tube, reduce the flame until the contents are just boiling and place a watch glass

over the mouth of the test tube. Continue heating for two minutes. The dark color will disappear, leaving about ½ c.c. of clear light blue solution in the tube. Cool for 70 to 90 seconds, and cautiously add 5 to 6 drops of water. After the violence of the mixing has subsided, add 20 c.c. of distilled water, and after cooling to about room temperature, add water to the 35 c.c. mark. Add 15 c.c. of Nessler's solution and mix. If turbidity develops, centrifuge. A white sediment (silicates) is not significant, but if yellow or brown, the determination must be repeated. If the nonprotein nitrogen is very high, use $^1/_2$ or $^1/_3$ the amount of filtrate. This must be compared with the *standard solution,* prepared as follows:

Standard ammonium sulphate solution.....	3.0 c.c.
Sulphuric-phosphoric acid mixture.....	2.0 c.c.
Distilled water to	60.0 c.c.
Nessler's solution (drop by drop).....	30.0 c.c.
Distilled water to	100.0 c.c.

Place the standard solution in the cup of a Duboscq or Bock-Benedict colorimeter and set at 20 mm. for color comparison. The unknown solution is placed in the other cup, and the colors are matched. Check up the colorimeter before the determination, by placing standard solution in both cups and comparing.

All glassware with which Nessler's solution has come in contact must be washed with nitric acid, and rinsed in distilled water.

Calculations:

$$\frac{\text{Reading of standard in mm.}}{\text{Reading of unknown in mm.}} \times 0.3 \times 100 = \text{Number of milligrams of nonprotein nitrogen in 100 c.c. of blood.}$$

If ½ the amount of filtrate has been used, multiply the result by 2; if $^1/_3$ was used, multiply by 3.

Example.

Reading of standard.....	20 mm.
Reading of unknown.....	24 mm.

$\frac{20}{24} \times 0.3 \times 100 = 25$ mg. of nonprotein nitrogen in 100 c.c. of blood.

BLOOD UREA NITROGEN [114]

Significance.—Urea is chiefly exogenous in origin and is formed largely in the liver from the ammonia resulting from the deaminization of amino-acids set free in digestion. *Normal* blood contains 12 to 20 mgm. of urea nitrogen (25 to 40 mgm. of urea) per 100 c.c. of blood. The urea nitrogen constitutes 50 per cent of the total nonprotein nitrogen. In cases of advanced *nephritis* and *uremia,* the percentage may rise to 75 or over.

In early *interstitial nephritis,* the urea nitrogen varies between 20 and 40. In advanced cases of this disease the values may rise to 460. Increases in the urea nitrogen content of the blood are also found in *bichlorid poisoning, double polycystic kidney, malignancy, pneumonia, intestinal obstruction, lead poisoning,* and occasionally in *syphilis* and *cardiac insufficiency.*

Reagents:

1. *Urease Solution.*

Wash 3 gms. of permutit in a 200 c.c. Erlenmeyer flask, *once* with 2 per cent acetic acid, then *twice* with water; add

Jack-bean meal	5.0 gms.
30 per cent alcohol	100.0 c.c.

Shake above contents for 10 to 15 minutes, then filter. The filtrate contains urease. This solution will last 2 to 4 weeks, if kept on ice. Urease powder (Arlington Chemical Co.) or urease tablets may be used instead of the above solution.

2. *Buffer Mixture.*

Monosodium phosphate	69.0 gms.
Crystallized disodium phosphate	179.0 gms.

Dissolve above in 800 c.c. of warm distilled water and then make up to one liter with distilled water.

3. *Hydrochloric Acid* $\frac{N}{20}$

Normal hydrochloric acid	5.0 c.c.
Distilled water to	100.0 c.c.

4. *Nessler's Solution* (p. 378).

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[114] Folin, O. and Wu, H. "A System of Blood Analysis." *Jour. Biol. Chem.,* 1919, XXXVIII, 81.

Method.—Introduce 5 c.c. of the Folin blood filtrate into a "pyrex" test tube (200 × 25 mm.). If the test tube has contained Nessler's solution, it must be rinsed first with nitric acid and then with water. Add 2 to 3 drops of buffer mixture, 1 c.c. of urease solution (or 100 mg. of urease powder or a urease tablet), immerse the tube in warm water, 50° to 55° C., and leave for 5 to 10 minutes.

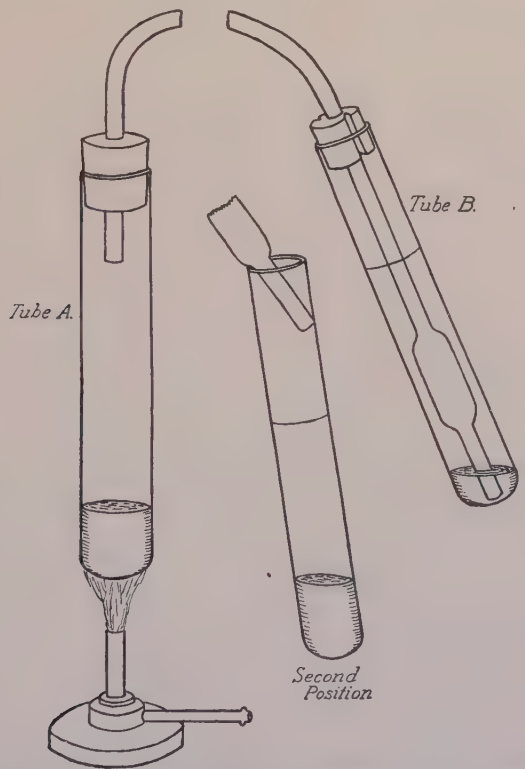

FIG. 95.—APPARATUS FOR DISTILLATION OF AMMONIA IN THE DETERMINATION OF UREA IN THE BLOOD (after Folin).

The ammonia formed from urea is obtained by distillation in an apparatus shown in illustration (Fig. 95). The ammonia vapor is allowed to bubble through $\frac{N}{20}$ hydrochloric acid solution.

Set up the apparatus as shown in illustration (Fig. 95), tube *A* containing the urease blood filtrate, tube *B* (a test tube graduated at 25 c.c.) containing 2 c.c. of $\frac{N}{20}$ hydrochloric acid, and stopper tube *A* tightly.

Lift the stopper from tube *A*, add 4 to 5 drops of paraffin oil, 1 c.c. of 10 per cent NaOH, and then restopper quickly and tightly so that one has a closed system, and any ammonia liberated in the cold will bubble through the $\frac{N}{20}$ hydrochloric acid solution. Insert a microburner beneath tube *A*, and heat at a uniform rate for 4 minutes, boiling for the last two minutes. Then lift the stopper from tube *B*. Arrange the apparatus as in the second position, and continue vigorous boiling for another minute. Rinse the lower end of the delivery tube *C* with distilled water into tube *B*, cool the contents of tube *B* and dilute to 20 c.c.

Preparation of the Standard Solution.—Transfer 3 c.c. of standard ammonium sulphate solution (0.3 mg. N) to a 100 c.c. volumetric flask and dilute to about 70 c.c. with distilled water. Add 10 c.c. of Nessler's solution and dilute to volume (100 c.c.).

Then add 2.5 c.c. of Nessler's solution to tube *B*. Dilute to volume (25 c.c.) and make the color comparison in the colorimeter.

Calculation:

$$\frac{\text{Reading of the standard in mm.}}{\text{Reading of the unknown in mm.}} \times 15 = \text{mg. of urea nitrogen per 100 c.c. of blood.}$$

If dilution of the filtrate is necessary, multiply the answer obtained above by the dilution to obtain the final answer.

Modified Urea Method.[115]—This method is especially applicable in cases where one wishes to make a series of urea determinations on a large number of blood specimens. The same reagents are required as for the Folin and Wu method.

Introduce 2 c.c. of oxalated blood into a tube previously washed with nitric acid and then with water; add 100 mg. of urease powder.[116] The tube is then immersed in warm water, 50° to 55° C., for 15 minutes. The tube is removed from the water bath, and the Folin filtrate is prepared by adding to the urease blood 14 c.c. of water, 2 c.c. of 10 per cent sodium tungstate solution and 2 c.c. of $^2/_3$ N sulphuric acid as described on page 371. When the contents of the tube assume a chocolate

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[115] A modification of the method described by Meyers (*loc. cit.*) as devised by the authors (I. and H.) and employed in the laboratory of the Cincinnati General Hospital. A similar method is described by Stitt.

urease powder. The urease powder has been found most satisfactory. If one c.c. of urease solution is used, then add 13 c.c. of water instead of 14 c.c.

brown color, filter. Place 5 c.c. of the filtrate in a tube graduated at 25 c.c., add 15 c.c. of water, nesslerize with 2.5 c.c. of Nessler's solution and dilute to volume (25 c.c.).

Prepare the standard solution simultaneously with the unknown by placing 3 c.c. (0.3 mg. N) of standard ammonium sulphate solution in a 100 c.c. volumetric flask, dilute to 70 c.c. with distilled water, add 10 c.c. of Nessler's solution and dilute to mark. *The standard and unknown solutions should be nesslerized as nearly simultaneously as possible.* Make color comparison in a colorimeter. The calculations are the same as with the Folin and Wu urea method.

THE AMINO-ACID NITROGEN IN THE BLOOD[117]

Significance.—The whole blood contains, after a night's fast, 5.7 to 7.8 mg. of amino-acid nitrogen per 100 c.c. of whole blood. (Av. 6.4 mg.). Next to the urea nitrogen, the amino-acid nitrogen is the second largest component of the nonprotein nitrogen. Its clinical significance is now being studied.[118]

Reagents:

1. *Standard amino-acid solution.*—This contains .07 mg. of nitrogen per c.c. To make 200 c.c., dissolve .075 gm. of pure glycine in about 150 c.c. of $\frac{N}{10}$ hydrochloric acid, add .4 gm. sodium benzoate and make up to 200 c.c. with $\frac{N}{10}$ hydrochloric acid. The solution will keep indefinitely. The glycine may be purified by dissolving in water and precipitating by the addition of ½ to 1 volume of alcohol.

2. *Special sodium carbonate solution.*—Fifty c.c. of approximately saturated sodium carbonate solution are diluted to 500 c.c. The strength of the solution is determined by titrating 20 c.c. of $\frac{N}{10}$ hydrochloric acid with the carbonate, using methyl red as indicator. On the basis of this value, the carbonate solution is diluted so that 8.5 c.c. are equivalent to 20 c.c. of $\frac{N}{10}$ acid. The carbonate solution is about 1 per cent.

3. *Five per cent solution of the sodium salt of B-napthoquinone sulphonic acid.*—This solution should always be freshly prepared. It is well to have several small flasks charged with 100 mg. of the salt, stored

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[117] Folin, O. and Wu, H. "A New Colorimetric Method for the Determination of the Amino-Acid Nitrogen in Blood." *Jour. Biol. Chem.*, 1922, LI, 377.

[118] Folin, O. and Berglund, H. "The Retention and Distribution of Amino-Acids with Especial Reference to the Urea Formation." *Jour. Biol. Chem.*, 1922, LI, 395.

in a dark place. As needed, 20 c.c. of water may be added to each. If the chemically pure salt is not obtainable, the pure substance may be made according to directions given by Folin.[119]

4. *Special acetic acid-acetate solution.*—Dilute 100 c.c. of 50 per cent acetic acid with 100 c.c. of 5 per cent sodium acetate solution.

5. *Four per cent solution of sodium thiosulphate.*—$Na_2S_2O_3$ $5H_2O$.

6. *Twenty-five hundredths per cent solution of phenolphthalein.*

Method.—Five or 10 c.c. of the tungstic acid filtrate (p. 371) may be used; the latter quantity is, however, to be recommended. (In the following, the *figures in parenthesis* indicate the *amounts of solutions to be taken where 10 c.c. of filtrate are used.*)

Five c.c. (or 10 c.c.) of the Folin and Wu filtrate are placed in a test tube of about 30 c.c. capacity. A 25 c.c. graduated test tube is convenient if 10 c.c. of filtrate are taken.

In a similar tube place 1 c.c. of the standard acid glycine solution (sol. 1) and 3 c.c. of water (8 c.c.). Add 1 drop of 0.25 per cent phenolphthalein solution to each. Add 1 c.c. of the 1 per cent sodium carbonate solution to the standard, and then add to the unknown carefully, drop by drop, sodium carbonate until it has approximately the same pink color (3 or 4 small drops usually required). Add another 5 c.c. of water to the standard. Prepare the 5 per cent sodium salt of B-napthoquinone sulphonic acid solution. Add 2 c.c. to the standard and 1 c.c. (2 c.c.) to the filtrate. Shake a little to make uniform and set aside in a completely dark cupboard until the following day, i. e., 19 to 30 hours.

Then add the acetic acid-acetate solution, 2 c.c. to the standard and 1 c.c. (2 c.c.) to the blood filtrate. Then add the thiosulphate solution 2 c.c. to the standard and 1 c.c. (2 c.c.) to the unknown. When 5 c.c. has been used, add 14 c.c. of water to the standard and 7 c.c. to the unknown with a "blood pipette." In case 10 c.c. were taken, dilute the standard and the unknown to 25 c.c. Mix and make the comparison in the colorimeter, setting the standard at 20.

Calculation:

$$\frac{20 \times 7}{\begin{matrix}\text{Reading}\\ \text{of}\\ \text{unknown}\end{matrix}} = \text{Amino-acid N in mg. per 100 c.c. blood.}$$

[119] Folin, O. and Wu, H. *loc. cit.*

Example:

Reading of standard..... 20 mm.
Reading of unknown..... 23.3 mm.

$\frac{20 \times 7}{23.3}$ = 6 mg. of amino-acid N in 100 c.c. of blood.

URIC ACID IN THE BLOOD [120]

Significance.—Uric acid originates as a result of the oxidation of the purines which are contained in the nuclear substances of the food and the tissues. This uric acid is both exogenous and endogenous in origin. *Normal* blood contains 2 to 4.0 mgm. of uric acid per 100 c.c. Uric acid increases following a diet rich in purines. Uric acid is the first of the nonprotein nitrogen constituents to increase in the blood in early cases of interstitial *nephritis.* In late cases of nephritis the uric acid may rise to 18 mg. In chronic cases of *gout* the blood uric acid may be within normal limits. It is decreased before the acute attacks, increasing with the onset, remaining above normal for a few days, then returning to normal. It is somewhat similar in cases of *rheumatoid arthritis.* A *purine-free diet* will lower the blood uric acid in cases of gout. *Phenylcinchoninic acid* (that is, "atophan," "cinchophen," etc.) and the *salicylates* induce an increased output of uric acid in the urine and a decreased concentration in the blood. The *toxemic vomiting of pregnancy* differs from the reflex vomiting in that blood uric acid is high in the former, normal in the latter.

Benedict's Method.—This method is simpler and gives more depth of color than the Folin-Wu procedure. Fewer reagents are required. The method does away with silver lactate precipitation and two centrifugations. It requires less blood than does any other uric acid determination. Benedict claims that an increased specificity of this procedure as regards uric acid is chiefly due to the reagent (arsenic-phosphoric acid-tungstic acid) used and partly to the use of cyanid instead of carbonate for development of the alkalinity.

The *normal* figures for this method are 2 to 4 mgm. per 100 c.c. of blood, about 0.5 mg. higher than those obtained by the Folin-Wu method.

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[120] Benedict, S. R. "The Determination of Uric Acid in Blood." *Jour. Biol. Chem.*, 1922, LI, 187; and Folin, O. and Wu, H.: "A System of Blood Analysis." *Jour. Biol. Chem.*, 1919. XXXVIII, 81.

Reagents:

1. *Phosphate standard uric acid solution* (stock).[121]

Pure crystallized disodium hydrogen phosphate.	9.0 gm.
Pure crystallized sodium dihydrogen phosphate.	1.0 gm.
Pure uric acid	200.0 mg.
Glacial acetic acid	1.4 c.c.
Distilled water to	1,000.0 c.c.
Chloroform (preservative)	5.0 c.c.

Make above solution as follows: Dissolve the disodium hydrogen phosphate and sodium dihydrogen phosphate in 200 to 300 c.c. of hot water. The solution is filtered, if it was not previously clear. The filtrate is made up to a total volume of about 500 c.c. with hot water, and this hot or warm (and perfectly clear) solution is poured upon exactly 200 mg. of pure uric acid suspended in a few c.c. of water in a liter volumetric flask. The mixture is agitated until the uric acid completely dissolves, and is then cooled. Exactly 1.4 c.c. of glacial acetic acid are added, and the contents of the flask are diluted to the mark and mixed. About 5 c.c. of chloroform are then added to prevent growth of bacteria or molds in the solution.

Five c.c. of this solution contains exactly 1 mg. of uric acid.

This standard stock solution will keep for about 2 months.

a. Phosphate standard uric acid solution, No. I (1 c.c. = 0.01 mg. uric acid).

Twenty-five c.c. of the phosphate standard stock solution (containing 5 mg. of uric acid) are measured into a 500 c.c. volumetric flask and the flask is about half filled with distilled water. Twenty-five c.c. of dilute hydrochloric acid (one volume of concentrated HCl diluted to 10 volumes with water) are added, and the solution is diluted to 500 c.c.

b. Phosphate standard uric acid solution, No. II (5 c.c. = 0.02 mg. of uric acid).

The procedure is the same, except that instead of beginning with 25 c.c. of the phosphate stock solution only 10 c.c. are used. This standard is the one most frequently used. These standards should be freshly prepared once every two weeks.

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[121] Benedict, S. R. and Hitchcock, E. H. "On the colorimetric estimation of uric acid in urine." *Jour. Biol. Chem.*, 1915, XX, 619.

2. *Arsenic-phosphoric acid-tungstic acid reagent.*
One hundred gm. of pure sodium tungstate are placed in a liter flask and dissolved in about 600 c.c. of water. Fifty gm. of pure arsenic pentoxide are now added, followed by 25 c.c. of 85 per cent phosphoric acid and 20 c.c. of concentrated hydrochloric acid. The mixture is boiled for 20 minutes, cooled, and diluted to 1 liter. The reagent appears to keep indefinitely.
This reagent yields nearly seven times as much color from a given weight of uric acid as does the old phosphoric acid reagent formerly employed and is probably more specific for uric acid than any of the reagents previously suggested.
3. *Five per cent sodium cyanid solution containing 2 c.c. of concentrated ammonia per liter.* It is desirable to prepare this solution freshly once in two months.

Method.—Transfer to one test tube (large enough to contain 30 c.c.) 5 c.c. of the Folin blood filtrate (p. 371) and to another 5 c.c. of the standard uric acid solution described above. Then to each tube add 5 c.c. of distilled water, 4 c.c. of 5 per cent sodium cyanid solution (from a burette) and 1 c.c. of the arsenic-phosphoric acid-tungstic acid reagent. Mix the contents of each tube thoroughly by inverting once, immediately after the addition of the last reagent. Then place them in a boiling water bath for 3 minutes, counting from the time the last tube is immersed and arranging so that the time elapsing between the immersion of the first and the last tube does not exceed 1 minute. Upon removal from the water bath, the tubes should be placed in a large beaker of cold water and allowed to stand for 3 minutes. Make the readings in a colorimeter within 5 minutes after the tubes have cooled. Turbidity may develop if the tubes stand too long before the reading is made.

Calculation.—Employing the standard solution containing 0.02 mg. of uric acid and using 5 c.c. of the blood filtrate, the calculation for the uric acid content of the original blood is as follows:

$$\frac{\text{Reading of standard (mm.)}}{\text{Reading of unknown (mm.)}} \times 4 = \text{mg. of uric acid per 100 c.c. of blood.}$$

If instead of using 5 c.c. of the blood filtrate in the determination, 2.5 or 10 c.c. are employed, the final figure is multiplied or divided by 2 accordingly.

Folin and Wu's Method.—Reagents:

1. *Standard uric acid-sulphite solution.*

In a 500 c.c. flask, dissolve.

Uric acid	1.0 g.
Distilled water	150.0 c.c.
Lithium carbonate	0.5 g.
Distilled water to	500.0 c.c.

Transfer 50.0 c.c. to a liter flask. Add

Distilled water to	1,000.0 c.c.
Sodium sulphite (20 per cent solution)	500 c.c.

Store in 200 c.c. bottles. A used bottle usually retains its strength for 2 to 3 months.

2. *Sodium sulphite*	10.0 g.
Distilled water to	100.0 c.c.
3. *Sodium cyanid*	5.0 g.
Distilled water to	100.0 c.c.
4. *Sodium chlorid*	10.0 g.
Hydrochloric acid (N/10) to	100.0 c.c.

5. *Uric acid reagent.*

Distilled water	750.0 c.c.
Sodium tungstate	100.0 g.
Phosphoric acid (85 per cent H_3PO_4)	80.0 c.c.

Partly close the mouth of the flask with a funnel and small watch glass; and boil gently for 2 hours. Cool and add

Distilled water to	1,000.0 c.c.
6. *Silver lactate*	5.0 g.
Lactic acid (5 per cent) to	100.0 c.c.
7. *Sodium carbonate* (anhydrous)	20.0 g.
Distilled water to	100.0 c.c.

Method.—To each of two centrifuge tubes add 10 c.c. of the Folin blood filtrate (p. 371), 2 c.c. of the silver lactate solution, and stir with a fine glass rod. A grayish white precipitate forms. Centrifuge. Test the clear supernatant solution with a drop of silver lactate solution.

It should remain perfectly clear, if precipitation has been complete. Decant the fluid part and discard it. Liberate the uric acid from the sediment by adding to each tube, 1 c.c. of the sodium chlorid-hydrochloric acid solution, and stir thoroughly. Add 5 to 6 c.c. water, stir, centrifuge. The supernatant fluid, containing the uric acid, should be transferred to a 25 c.c. volumetric flask (the unknown).

Prepare the standard solutions in two 50 c.c. volumetric flasks, placing 1 c.c. of the standard uric acid-sulphite solution in one flask and 2 c.c. in the other.

Then prepare the solutions simultaneously as follows:

Steps in Procedure

Unknown flask	Standard No. 1 flask	Standard No. 2 flask
1. 1 c.c. 10% sodium sulphite	1 c.c. 10% sodium sulphite	
2.	4 c.c. acid sodium chlorid solution	4 c.c. acid sodium chlorid solution
3. 0.5 c.c. sodium cyanid solution	1 c.c. sodium cyanid solution	1 c.c. sodium cyanid solution
4. 3 c.c. 20% sodium carbonate solution	6 c.c. 20% sodium carbonate solution	6 c.c. 20% sodium carbonate solution
5.	Water, to about 45 c.c.	Water, to about 45 c.c.
6. 0.5 c.c. uric acid reagent	1 c.c. uric acid reagent	1 c.c. uric acid reagent

Allow the color to develop for 10 minutes.

Then fill the three flasks to the graduated mark with water. Mix. Compare the color in the colorimeter.

Calculations:

Standard No. 1 (weaker).

$$\frac{\text{Reading of standard (mm.)}}{\text{Reading of unknown (mm.)}} \times 2.5 = \text{Number of milligrams of uric acid in 100 c.c. of blood.}$$

Standard no. 2 (stronger). Multiply the above by 2.

Example:

Standard set at 20. Standard Number 1 used.
Reading of unknown 25 mm.

$$\frac{20 \times 2.5}{25} = 2 \text{ mg. of uric acid per 100 c.c.}$$

PREFORMED CREATININ [122]

Significance.—The *normal* creatinin in the blood is 1 to 2 mg. per 100 c.c., with a maximum of 3.5 mg. It is practically independent of the amount of protein in the food. Retention is noted in *syphilis,* in *myocardial insufficiency,* in some cases of advanced *diabetes* (3 to 4 mg.) and in *nephritis. In nephritis, a persistent value of 5 mg. or above indicates a fatal prognosis.* Creatinin is the last of the group (uric acid, urea, creatinin) to accumulate in the blood, when renal function is impaired.

Reagents:

1. *Picric acid* (purified)—saturated solution.
2. *Sodium hydroxide*—10 per cent solution.

A saturated solution of sodium hydroxid is made and allowed to stand for a week or more. The supernatant fluid is approximately 50 to 55 per cent. Dilute the supernatant fluid with four volumes of water, and determine strength by titration with an acid of known strength (preferably 2N H_2SO_4).

3. *Stock creatinin solution.*

Creatinin zinc chlorid	1.61 gm.
Hydrochloric acid N/10 to	1,000 c.c.
Toluene	4 to 5 drops

To make up the standards a dilution of this solution is used as follows:

Stock solution	6.0 c.c.
Normal hydrochloric acid	10.0 c.c.
Water to	1,000.0 c.c.

[122] Folin, O. and Wu, H. *Loc. cit.,* p. 385.

Method.—To 25 c.c. of saturated solution of picric acid, add 5 c.c. of 10 per cent sodium hydroxid, and mix in a small flask. To a test tube, add 10 c.c. of the blood filtrate (p. 371). In another tube the standard is prepared as follows:

a. For normal blood—5 c.c. of the diluted stock plus 15 c.c. of water.

b. For bloods with high creatinin—10 c.c. of the diluted stock solution plus 10 c.c. of water.

c. Fifteen c.c. of the diluted stock solution plus 5 c.c. of water; or,

d. Twenty c.c. of diluted stock solution itself may be used, when the creatinin is expected to be above 6 mg. per 100 c.c. of blood.

Five c.c. of the freshly prepared picric acid—sodium hydroxid solution is then added to the test tube containing the filtrate, and 10 c.c. to the standard solution. After 8 to 10 minutes (within 15 minutes), the color comparisons are made in the colorimeter.

Calculations:

$$\frac{\text{Reading of standard (mm.)}}{\text{Reading of unknown (mm.)}} \text{ (usually 20) multiplied by}$$

1.5 (if the standard *a* is used; 3 for standard *b*; 4.5 for standard *c* and 6 for standard *d*), gives the number of mg. of creatinin in 100 c.c. of blood.

Example:

Reading of standard..... 20 mm.
Reading of unknown..... 15 mm.
Standard *a* used.

$$\frac{20 \times 1.5}{15} = 2 \text{ mg. of creatinin per 100 c.c. of blood.}$$

CHLORIDES IN BLOOD [123]

Significance.—The *normal* chlorid content of *whole blood,* in terms of sodium chlorid, is from 0.45 to 0.52 grams per 100 c.c. In the *plasma* the amount is from 0.57 to 0.62 grams per 100 c.c. The amount is

[123] Whitehorn, J. C. "A Simplified Method for the Determination of Chlorids in Blood or Plasma." *Jour. Biol. Chem.*, 1921, XLV, 449.

increased in certain *cardiac conditions,* in *anemia,* in some cases of *malignancy,* and in *nephritis.* In *chronic interstitial nephritis* the blood chlorid concentration can be controlled by diet, and the chlorid output does not vary as much from normal as in the *parenchymatous type.* The determination of the blood chlorids serves as a check on the necessity of chlorid restriction in the diet in nephritis. The amount is *decreased* in *fevers, diabetes* and *pneumonia.* In the latter disease the chlorid output is associated with a low concentration in the blood, differing from nephritis, when the low output is associated with accumulation in the blood.

Reagents:

1. *Silver nitrate solution* (M/35.46).

Silver nitrate (c.p.)	4.791	gm.
Distilled water to	1,000.0	c.c.

Mix thoroughly and preserve in a brown bottle. 1 c.c. = 1 mg. Cl.

2. *Potassium (or ammonium) sulphocyanate* (M/35.46).

Dissolve about 3 gm. of potassium sulphocyanate or 2.5 gm. of ammonium sulphocyanate in a liter of water. By titration and proper dilution prepare a standard so that 5 c.c. are equivalent to 5 c.c. of the silver nitrate solution.

3. *Powdered ferric ammonium sulphate* ($FeNH_4(SO_4)_2$).

4. *Concentrated nitric acid* (HNO_3 of specific gravity 1.42).

Method.—Pipette 10 c.c. of the Folin blood filtrate (p. 371) into a porcelain dish.

Add with a pipette 5 c.c. of the standard silver nitrate solution and stir thoroughly. Add about 5 c.c. of concentrated nitric acid, mix and let stand for 5 minutes, to permit the flocking out of the silver chlorid. Then add about 0.3 gm. of ferric ammonium sulphate and titrate the excess of silver nitrate with the standard sulphocyanate solution, until the definite salmon-red color of the ferric sulphocyanate persists, in spite of stirring for at least 15 seconds.

Calculation:

5.00 minus the titer (in c.c.) $\times$ 100 = mg. of chlorin per 100 c.c. of blood.

To convert the chlorin figures into sodium chlorid divide by 0.606.

Example:

Assuming the titer to be 2.35 c.c., then $5.00 - 2.35 = 2.65$ mg. of Cl. per c.c. of blood.

$2.65 \div 0.606 \times 100 = 437.29$ mg. of NaCl per 100 c.c. of blood.

FATTY ACIDS IN THE BLOOD [124]

Significance.—The *normal total fat content* of the blood plasma is 600 to 700 mg. per 100 c.c. The amount is increased after a *diet high in lipoids*, and in *diabetes*. In mild and moderately severe diabetes it may be doubled, and in severe cases may be three or more times the normal, varying from 0.3 per cent to 9.4 per cent in the post-absorptive state, reaching 26 per cent under some conditions. In *beriberi* the fat content may be reduced to one-third normal.

Reagents:

1. *Alcohol-ether mixture.*

Alcohol, absolute, redistilled	300.0 c.c.
Ether, redistilled	100.0 c.c.

2. *Sodium hydroxid solution.*

Expose a piece of metallic sodium to water vapor from distilled water, (in a closed vessel) at room temperature. The sodium hydroxid which drips off of the metal is caught in a receiver. The sodium may be placed on top of an inverted watch glass, placed in an evaporating dish, and the dish placed in a desiccator which contains distilled water in the bottom.

3. *Dilute sulphuric acid.*

Sulphuric acid, concentrated	25.0 c.c.
Water	75.0 c.c.

4. *Standard fat solution.*

a. Oleic acid stock solution.

Oleic acid	200 gm.
Alcohol (95 per cent redistilled)	500 c.c.

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[124] Bloor, W. R., Pelkan, K. F. and Allen, D. M. "Determination of Fatty Acids (and Cholesterol) in Small Amounts of Blood Plasma." *Jour. Biol. Chem.*, 1922, LII, 191.

b. Palmitic acid stock solution.

Palmitic acid	200 mg.
Alcohol (95 per cent redistilled)	500 c.c.

c. Oleic acid (stock solution)	60 c.c.
Palmitic acid (stock solution)	40 c.c.

Five c.c. of this solution contains 2 mg. of a mixture of the fatty acids.

5. *Diluted hydrochloric acid.*

Hydrochloric acid (concentrated)	25 c.c.
Water	75 c.c.

Method.—Fifteen to 20 c.c. of oxalated blood is centrifuged and 5 c.c. of the plasma is measured into a 100 c.c. flask containing about 75 c.c. of the alcohol-ether mixture. Large aggregates of precipitate are avoided by dropping the plasma slowly while rotating the flask rapidly. The flask is then immersed in boiling water, agitating to prevent superheating, until the liquid begins to boil. Cool to room temperature, add the alcohol-ether mixture to the 100 c.c. mark, mix and filter.

Ten to 20 c.c. of the filtrate (representing about 2 mg. of fatty acid) is measured into a 50 to 100 c.c. "Non-Sol" glass Erlenmeyer flask. Sodium hydroxid, 0.1 c.c. of the concentrated solution, is added, and the mixture evaporated on a water bath, with occasional shaking to distribute the liquid evenly over the bottom. The drying is continued until only 2 or 3 drops of liquid remain, and the odor of alcohol is entirely gone.

The alkali is then partially neutralized with 0.1 c.c. of the dilute sulphuric acid solution. Mix thoroughly and distribute over the bottom of the flask. If there is not enough liquid in the flask to allow complete mixing, add a drop or two of distilled water. Dry carefully on the water bath until all moisture has disappeared, avoiding overheating.

The cholesterol is then separated as follows: After cooling, 10 c.c. of chloroform (neutral, and free from moisture or alcohol) are added, and the flask allowed to stand for 10 minutes, with occasional shaking to allow the solvent to reach all the material. The chloroform extract is poured through a 5½ cm. hardened filter paper into another flask,

and the extraction twice repeated with 5 c.c. of chloroform, using care not to break the salt loose from the bottom of the original flask. The combined chloroform extract is used for determination of cholesterol (p. 396).

The fatty acids are determined from the residue in the small flask after the chloroform extraction. Ten c.c. of redistilled alcohol are added to each flask, the mixture is raised to boiling on an electric stove or water bath and kept boiling gently for 10 minutes. The hot alcohol is then poured through the small hardened filter which was used in filtering the chloroform, into a 100 c.c. Erlenmeyer flask. Extract the residue once more with 5 c.c. of alcohol, pouring it through the same filter. Evaporate the combined filtrates to 2 or 3 c.c. Transfer to a small, glass-stoppered cylinder, and rinse the flask with just enough alcohol to bring the volume in the cylinder up to 5 c.c. Measure 100 c.c. of distilled water into a 200 c.c. beaker and stir the alcoholic extract into it, with the stem of a funnel drawn out to an opening diameter of 1 mm. The tip of the funnel should extend nearly to the bottom of the beaker. The cylinder is rinsed once with the solution in the beaker, and the rinsings are poured back into the beaker, through the funnel.

To another beaker containing 100 c.c. of water, add through a pipette, with stirring, 5 c.c. of the standard fat solution. Add 10 c.c. of the dilute hydrochloric acid solution to each beaker with stirring. The solutions are then compared in the nephelometer after 3 minutes (not more than 10 minutes), using care to avoid bubbles.

Calculations.—Since the amount of suspensoids is not exactly inversely proportional to the readings on the nephelometer scale, it is necessary to determine the ratio of concentrations by calculating from a formula.

If the depths of the tubes are from 50 to 60 mm. and the concentrations of the unknown and standard are within 20 per cent of each other, fairly accurate results, however, may be obtained directly. With a shorter tube the results are accurate only if the difference between the unknown and standard are within 10 per cent.

Calculations

$$\frac{\text{Reading of standard (mm.)}}{\text{Reading of unknown (mm.)}} \times \frac{\text{Amount of fatty acid in standard}}{\text{Volume of blood}} \times 100 =$$

Number of mg. of fatty acids in 100 c.c. blood.

Example

Reading of standard	30 mm.
Reading of unknown	25 mm.
Amount of fatty acid in standard	2 mg.
Volume of blood represented	.5 c.c.

$$\frac{30}{25} \times \frac{2.0}{.5} \times 100 = 480 \text{ mg. of fatty acid per 100 c.c. blood.}$$

CHOLESTEROL IN THE BLOOD [125]

Significance.—*Normal whole blood* contains from 0.14 to 0.17 grams of cholesterol per 100 c.c. The *serum* contains 0.15 to 0.18 grams. The amount may vary with the cholesterol and fat content of the diet. Low values are found in *pernicious anemia* (0.07) and in *high fevers.* It is increased in *acute and chronic nephritis* (0.17 to 0.35), *diabetes* (0.15 to 0.30), *lipemia* (to 3.6) and in *pregnancy, arteriosclerosis,* and *complete obstruction of common bile duct* (cholelithiasis). It is low or normal in *hemolytic jaundice.*

Reagents:

1. *Chloroform* (neutral, and free from moisture and alcohol).
2. *Stock cholesterol solution.*

Cholesterol	0.2 gms.
Chloroform to	200.0 c.c.

3. *Standard cholesterol solution.*

Stock solution (see above)	10.0 c.c.
Chloroform to	100.0 c.c.

4. *Acetic anhydrid.*
5. *Sulphuric acid,* concentrated.

Method.—The chloroform extract obtained in the extraction of blood (p. 394) is transferred to a glass-stoppered 10 c.c. cylinder, and the

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[125] Bloor, W. R. "The Determination of Cholesterol in Blood." *Jour. Biol. Chem.*, 1916, XXIV, 227.

Bloor, W. R., Pelkan, K. F. and Allen, D. M. "Determination of Fatty Acids (and Cholesterol) in Small Amounts of Blood Plasma." *Jour. Biol. Chem.*, 1922, LII, 191.

volume made up to 5 c.c. with chloroform. The solution will be colorless, although not necessarily clear.

Measure 5 c.c. of the standard cholesterol solution into a similar 10 c.c. cylinder.

To the standard and to the unknown solutions add 1 c.c. acetic anhydrid and 0.1 c.c. concentrated sulphuric acid. Mix, by inverting several times, then set aside for 15 minutes at 15° to 20° C. exposed to the same light by which the readings are to be made. The solutions are then compared in the colorimeter, setting the standard at 15 mm. (The cement of the colorimeter cups must be insoluble in chloroform. Plaster of Paris or glue is satisfactory.)

Calculations

$$\frac{\text{Reading of standard (mm.)}}{\text{Reading of unknown (mm.)}} \times \frac{\text{Amount of cholesterol in standard}}{\text{Actual volume of blood used}} \times 100 =$$

Number of mg. of cholesterol in 100 c.c. blood.

Example

Reading of standard	15 m.m.
Reading of unknown	12 m.m.
Amount of cholesterol in standard	.5 mg.
Volume of blood represented	.5 c.c.

$$\frac{15}{12} \times \frac{.5}{.5} \times 100 = 125 \text{ mg. of cholesterol per 100 c.c. of blood.}$$

THE CARBON-DIOXIDE COMBINING POWER OF BLOOD PLASMA ("ALKALI RESERVE")

Significance.[126]—The *normal* carbon dioxid combining power of the plasma varies from 53 to 77 c.c. of gas liberated from 100 c.c. of plasma. In *infants* the numbers vary from 40 to 55. Percentages lower than 50 in adults indicate *"acidosis,"* symptoms of acid intoxication appearing when the number falls below 30.

Since the determination of the carbon dioxid combining power gives a measure of the alkali carbonates available for the neutralization of

[126] Van Slyke, D. and Cullen, E. "The Bicarbonate Concentration of the Blood Plasma; its Significance, and its Determination as a Measure of Acidosis." *Jour. Biol. Chem.*, 1917, XXX, 289.

Van Slyke, D. *Ibid.*, p. 347.

acids, it is frequently referred to as the *alkali reserve.* In *diabetes,* a low number often gives a clue of impending coma, and the alkali reserve, which may drop as low as 10, is a measure of the increased acid production. In *nephritis,* on the other hand, acids may tend to accumulate because of poor excretion, and in acute nephritis numbers from 45 to 20 have been encountered; the reserve may drop as low as 12 in the terminal stages of chronic interstitial nephritis. The findings are not necessarily low in *uremia.* In *epidemic influenza* and *broncho-pneumonia,* a persistently low alkali reserve, below 46, indicates bad prognosis. In *diarrhea* in children a low reserve (13 to 38) may be encountered. The alkali reserve is depleted after chloroform or ether *anesthesia,* and to a lesser degree after gas oxygen inhalation. The administration of alkali salts of weak or easily oxidized acids increases the reserve.

Reagents:

1. *Sulphuric acid* $\frac{N}{1}$
2. *Octyl alcohol: Capryllic alcohol.*
3. *Ammonium hydroxid* (dilute).

Method.—Five to 10 c.c. of blood, collected under the special precaution indicated on p. 371, are centrifuged immediately. Two to 3 c.c. of

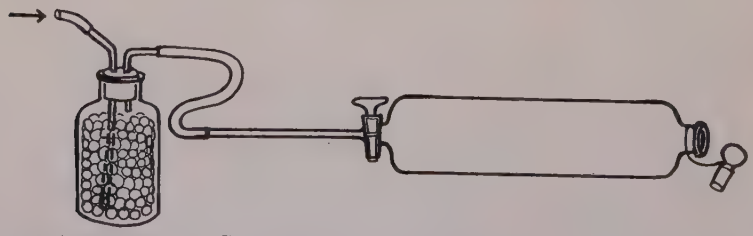

Fig. 96.—Apparatus for Saturating the Blood Plasma with CO_2 at Alveolar Tension (after Van Slyke).

the plasma are transferred to a 300 c.c. separatory funnel and a stream of air from the lungs (one expiration) blown through. This saturates the plasma with carbon dioxid at alveolar tension. To prevent condensation of the exhaled moisture, the air is blown through a bottle containing glass splinters or beads, arranged as in the diagram (Fig. 96). Thorough mixture is obtained by rotating the funnel, and distributing the plasma over the walls. The process may be repeated once more to insure saturation.

The apparatus (Fig. 97), including both capillaries of the upper stop-cock, is filled with mercury. With the upper stop-cock closed, the mer-

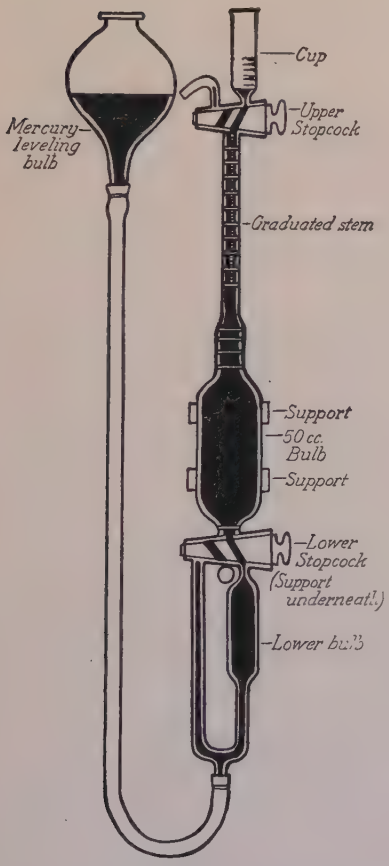

Fig. 97.

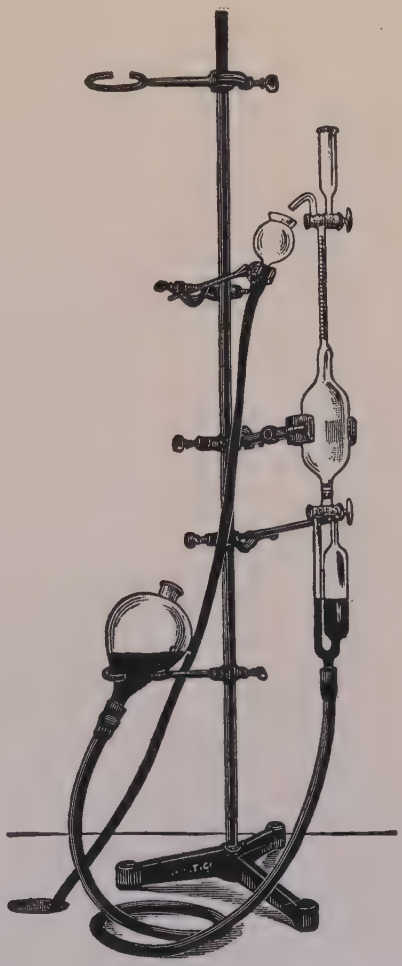

Fig. 98.

Fig. 97.—Apparatus for the Determination of the Carbon Dioxide Combining Power of Blood Plasma.
Fig. 98.—The Van Slyke Apparatus Mounted on Stand.

cury leveling bulb is lowered several times to extract the air from the apparatus and to detect leakage. Imprisoned air is released through the

upper stop-cock. When no more air is present, the mercury will return with a *snap,* otherwise the sound is dull.

With the leveling bulb at the level of the lower stop-cock, the cup is washed out with dilute ammonia, followed by water. One cubic centimeter of the saturated plasma is introduced into the bottom of the cup, and the stop-cock opened slightly, so that the plasma enters the narrow graduated stem. The cup is then washed with two portions of water (½ c.c. each). To prevent foaming, one drop of octyl alcohol is allowed to flow into the capillary tube. *Normal* sulphuric acid (2 c.c.) is put into the cup and allowed to run into the graduated stem, until the total quantity of fluid above the mercury reaches the 2.5 c.c. mark. The capillary bore of the upper stop-cock is then sealed by allowing a little mercury to run into it and the supernatant fluid pipetted off.[127] A rubber stopper is then inserted into the mouth of the cup.

The leveling bulb is then lowered until the level of the fluid has dropped to the 50 c.c. mark.

The carbon dioxid and dissolved air will escape as the vacuum is produced. The apparatus is then removed from the clamp (Fig. 98), and the contents inverted fifteen times. The apparatus is remounted and, with the leveling bulb lowered, the lower stop-cock is so turned that the fluid runs from the 50 c.c. chamber into the small bulb below the stop-cock. When the fluid has been drained completely, the gas is imprisoned in the 50 c.c. bulb. A turn of the stop-cock will then allow the mercury to run from the narrower tube, which runs parallel to the small bulb, into the gas chamber, when the leveling bulb is raised. Pressure is equalized when the mercury in the bulb and the gas chamber are at the same level. The reading is taken from the *top* of the thin layer of fluid, which usually collects above the mercury.

To clean the apparatus, allow the fluid to run back into the 50 c.c. bulb and then into the cup, from which it can be pipetted off, or else it can be drained from the side tube leading from the upper stop-cock.

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[127] A modification in use in the laboratory at the Cincinnati General Hospital is as follows: Two drops of caprillic alcohol are put into the cup. One c.c. of the saturated plasma is allowed to run beneath the oily layer, from a pipette. The stop-cock is slowly turned until the plasma runs into the stem of the apparatus. The line of contact of the caprillic alcohol and the plasma is clear cut and can be followed until the plasma has all entered the stem. The sulphuric acid is allowed to run in, in the same way. The extra fluid is pipetted from the cup, and the capillary tube sealed with mercury.

The inside of the apparatus can then be washed with a little dilute ammonium hydroxid.

Calculations.—The observed reading is corrected for the gas mechanically dissolved in the 2.5 c.c. of solution used, and the volume of gas is reduced to 0° C. and 760 mm. pressure. This can be calculated from the tables. For clinical purposes, sufficiently accurate results may be obtained if .12 is subtracted from the observed reading. This number multiplied by 100 gives the volume per cent of CO_2 chemically bound by the plasma.

$$\text{Observed reading corrected for temperature} \times \frac{\text{Barometric reading}}{760}$$

can be calculated from the tables. $\frac{\text{Barometric reading}}{760}$ can be calculated from the first table. The second table gives the c.c. of carbon dioxid, reduced to 0° C. bound as bicarbonate in 100 c.c. of plasma.

TABLE I

FACTORS FOR CORRECTION FOR BAROMETRIC PRESSURE

Barometer	$\frac{B}{760}$	Barometer	$\frac{B}{760}$
732	0.963	756	0.995
734	0.966	758	0.997
736	0.968	760	1.000
738	0.971	762	1.003
740	0.974	764	1.006
742	0.976	766	1.008
744	0.979	768	1.001
746	0.981	770	1.013
748	0.984	772	1.016
750	0.987	774	1.018
752	0.989	776	1.021
754	0.992	778	1.024

TABLE II

FOR CALCULATION OF CARBON DIOXIDE COMBINING POWER IN PLASMA

Observed Vol. Gas $\times \frac{B}{760}$ *	C.c. of CO_2 reduced to 0°, 760 mm., bound as bicarbonate by 100 c.c. of plasma				Observed Vol. Gas $\times \frac{B}{760}$	C.c. of CO_2 reduced to 0°, 760 mm., bound as bicarbonate by 100 c.c. of plasma			
	15°	20°	25°	30°		15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
0.21	10.1	10.9	11.7	12.6	0.61	48.7	49.0	49.4	49.5
0.22	11.0	11.8	12.6	13.5	0.62	49.7	50.0	50.4	50.4
0.23	12.0	12.8	13.6	14.3	0.63	50.7	51.0	51.3	51.4
0.24	13.0	13.7	14.5	15.2	0.64	51.6	51.9	52.2	52.3
0.25	13.9	14.7	15.5	16.1	0 65	52.6	52.8	53.2	53.2
0.26	14.9	15.7	16.4	17.0	0.66	53.6	53.8	54.1	54.1
0.27	15.9	16.6	17.4	18.0	0.67	54.5	54.8	55.1	55.1
0.28	16.8	17.6	18.3	18.9	0.68	55.5	55.7	56.0	56.0
0.29	17.8	18.5	19.2	19.8	0.69	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
0.31	19.7	20.4	21.1	21.7	0.71	58.4	58.6	58.9	58.8
0.32	20.7	21.4	22.1	22.6	0.72	59.4	59.5	59.8	59.7
0.33	21.7	22.3	23.0	23.5	0.73	60.3	60.5	60.7	60.6
0.34	22.6	23.3	24.0	24.5	0.74	61.3	61.4	61.7	61.6
0.35	23.6	24.2	24.9	25.4	0.75	62.3	62.4	62.6	62.5
0.36	24.6	25.2	25.8	26.3	0.76	63.2	63.3	63.6	63.4
0.37	25.5	26.2	26.8	27.3	0.77	64.2	64.3	64.5	64.3
0.38	26.5	27.1	27.7	28.2	0.78	65.2	65.3	65.5	65.3
0.39	27.5	28.1	28.7	29.1	0.79	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
0.41	29.4	30.0	30.5	31.0	0.81	68.1	68.1	68.3	68.0
0.42	30.3	30.9	31.5	31.9	0.82	69.0	69.1	69.2	69.0
0.43	31.3	31.9	32.4	32.8	0.83	70.0	70.0	70.2	69.9
0.44	32.3	32.8	33.4	33.8	0.84	71.0	71.0	71.1	70.8
0.45	33.2	33.8	34.3	34.7	0.85	71.9	72.0	72.1	71.8
0.46	34.2	34.7	35.3	35.6	0.86	72.9	72.9	73.0	72.7
0.47	35.2	35.7	36.2	36.5	0.87	73.9	73.9	74.0	73.6
0.48	36.1	36.6	37.2	37.4	0.88	74.8	74.8	74.9	74.5
0.49	37.1	37.6	38.1	38.4	0.89	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
0.51	39.1	39.5	40.0	40.3	0.91	77.8	77.7	77.7	77.3
0.52	40.0	40.4	40.9	41.2	0.92	78.7	78.8	78.7	78.2
0.53	41.0	41.4	41.9	42.1	0.93	79.7	79.6	79.6	79.2
0.54	42.0	42.4	42.8	43.0	0.94	80.7	80.5	80.6	80.1
0.55	42.9	43.3	43.8	43.9	0.95	81.6	81.5	81.5	81.0
0.56	43.9	44.3	44.7	44.9	0.96	82.6	82.5	82.4	82.0
0.57	44.9	45.3	45.7	45.8	0.97	83.6	83.4	83.4	82.9
0.58	45.8	46.2	46.6	46.7	0.98	84.5	84.4	84.3	83.8
0.59	46.8	47.1	47.5	47.6	0.99	85.5	85.3	85.2	84.8
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

* B = Barometric pressure, (mm.).

THE OXYGEN COMBINING POWER OF THE BLOOD [128]

Significance.—The *normal* oxygen combining power of the blood, under standard conditions of temperature and pressure, is 20.9 c.c. per 100 c.c. of blood when the hemoglobin is 100 per cent, varying from 14.42 to 22.08 c.c. as the hemoglobin varies. The oxygen capacity falls slightly after exercise. The oxygen combining power can be used as a basis for calculating the percentage of hemoglobin present, 20.9 c.c. of oxygen corresponding to 100 per cent of hemoglobin (see p. 264). If extreme care is used in collecting the blood (under oil, and without suction), the *oxygen content* of the venous and arterial blood can be determined by the method described below. From the total oxygen combining power and the actual oxygen content of the blood, the percentage of oxygen *unsaturation* can be calculated. *Normally,* the arterial blood is about 85 to 98 per cent saturated. In decompensated *circulatory disturbances* the capacity varies from 16.33 to 28.85 volumes per cent. In nonfatal cases of *pneumonia* it is 14.9 to 26.3 (av. 20.0) volumes per cent, and in fatal cases with intense cyanosis 16.2 to 28.6 volumes per cent; it is 32 per cent saturated in influenzal *bronchopneumonia* 12 to 24 hours before death.

Reagents:

1. *Octyl alcohol.*
2. *One per cent saponin.*
3. *Twenty per cent potassium ferricyanid solution.* This must be air-free. It can be made so by boiling. It may be kept in a burette covered by a layer of paraffin oil 2 or 3 cm. thick.

Method.—The following method is a modification of the Van Slyke-Stadie method suggested by Lundsgaard and Möller.

The Van Slyke carbon dioxid apparatus (p. 399) is employed in the determination which is carried out as follows: Six c.c. of water containing 2 to 3 drops of octyl alcohol and .3 c.c. of 1 per cent saponin are run into the apparatus. The chamber is evacuated as in the carbon dioxid determination, and the air extracted from the water by shaking

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[128] Van Slyke, D. D. "Gasometric Determination of the Oxygen of Blood." *Jour. Biol. Chem.*, 1918, XXIII, 127. Van Slyke, D. D., and Stadie, W. C. "The determination of the gases of the blood." *Jour. Biol. Chem.*, 1921, XLIX, 1. Lundsgaard, C. and Möller, E. "On the Determination of the Total Oxygen Combining Power of the Blood in the Van Slyke Apparatus." *Jour. Biol. Chem.*, 1922, LII, 377.

15 seconds. The air is expelled and the extraction repeated to make sure that no air is left in solution. The liquid is then trapped in the wide branch of the apparatus below the lower stop-cock. The stop-cock is turned and the mercury is run very slowly upwards through the apparatus in order to collect the film of water left on the inside. The water is then run out of the left side of the upper part. The upper stop-cock is now turned and the mercury is run into the bottom of the cup. If any moisture is left in the cup from the introduction of water it is dried by filter paper. Two c.c. of blood are now introduced into the cup and drawn down almost to the bottom of the 50 c.c. chamber. The apparatus is now shaken ½ minute by hand or 2 to 3 minutes by a mechanical shaker. The upper stop-cock remains open. Thereby the blood is saturated. Mercury is again run up into the 50 c.c. chamber collecting the blood at the top. When the blood column reaches the upper stop-cock, this is closed. The stop-cock is now turned so that the previously trapped air-free water is allowed to rise into the chamber.

The lower stop-cock is closed and the apparatus turned upside down once in order to mix the blood and water. After ½ minute the blood is laked. One-tenth c.c. of the 20 per cent air-free potassium ferricyanid is run in. The apparatus is evacuated by lowering the leveling bulb until only a few drops of mercury remain above the lower stop-cock, and is shaken, preferably in a rotary motion, to whirl the blood in a thin layer around the wall of the chamber. If the blood was completely laked before the cyanid was added, extraction of the oxygen may be completed by half a minute of efficient shaking. The lower stop-cock is now opened and the levels of mercury adjusted, the reading being then taken. To insure that all the oxygen has been liberated, the apparatus is evacuated once more and the blood shaken again for ½ minute. The second reading should equal the first. It is important to wash out the apparatus after using.

Calculation.—In order to calculate the volume of oxygen bound by the hemoglobin it is necessary to subtract from the gas measured the volume of air physically bound by the 2 c.c. of blood at atmospheric pressure and room temperature. The volume of gas thus corrected may be reduced to standard conditions by multiplying by $(.999 - .0046\ t) \times \frac{\text{barometer}}{760}$, t being the temperature in degrees centigrade.

The following table is from Van Slyke.

TABLE III

FACTORS FOR CALCULATING RESULTS FROM ANALYSIS OF 2 C.C. OF BLOOD SATURATED WITH AIR

Temp.	Air physically dissolved by 2 c.c. blood. Subtract from gas volume read in order to obtain corrected gas volume, representing O_2 set free from hemoglobin	Factor by which corrected gas volume is multiplied to give	
		Oxygen chemically bound by 100 c.c. blood	Per cent hemoglobin calculated on the basis of 20.9 per cent oxygen, corresponding to 100 per cent. hemoglobin.
C. °	c.c.	c.c.	Per cent
15	0.037	$46.5 \times \frac{B}{760}$ *	$222 \times \frac{B}{760}$
16	0.036	46.3 "	221 "
17	0.036	46.0 "	220 "
18	0.035	45.8 "	219 "
19	0.035	45.6 "	218 "
20	0.034	45.4 "	217 "
21	0.033	45.1 "	216 "
22	0.033	44.9 "	214 "
23	0.032	44.7 "	213 "
24	0.032	44.4 "	212 "
25	0.031	44.2 "	211 "
26	0.030	44.0 "	210 "
27	0.030	43.7 "	209 "
28	0.029	43.5 "	208 "
29	0.029	43.3 "	207 "
30	0.028	43.1 "	206 "

* B = Barometric reading.

Example.—The following example illustrates a calculation:

Observed gas volume, at 20°, 750 mm.....	0.450 c.c.
Correction for dissolved air.....	.034 c.c.
Corrected gas volume.....	.416 c.c.

$.416 \times 44.8 = 18.65$ volume.

$.416 \times 243 = 101$ per cent hemoglobin.

CHAPTER IX

PUNCTURE FLUIDS

The methods described below are applicable to most puncture fluids, e. g., pleural, peritoneal, pericardial, hydrocele, etc. The examination of the cerebrospinal fluid, however, requires certain special procedures, which are considered separately.

It is important, especially in pleural, pericardial and peritoneal fluids, to differentiate between inflammatory exudates and transudates.

TABLE SHOWING THE DIFFERENCES BETWEEN EXUDATES AND TRANSUDATES OCCURRING IN PLEURA, PERICARDIUM AND PERITONEUM

Criterion	*Exudate*	*Transudate*
Nature of pathological lesion	Inflammatory	Noninflammatory
Specific gravity	1.018 or higher.	1.015 or lower.
Protein per liter	30 to 70 gms.	Less than 30 gms.
Cells	Lymphocytes usually predominate in serous exudates, often amounting to 90 per cent of the cells. Endothelial cells are always present. There are always a few polynuclear neutrophiles. As the exudate becomes purulent, pus cells are more and more numerous. At times, eosinophiles and basophiles are conspicuous. Red blood cells may exist in the exudate or may be present as a result of the puncture wound.	Endothelial cells and lymphocytes are found. Red blood corpuscles, when present, are the result of the puncture wound. Fluid is serous.
Bacteria	Usually demonstrable by culture or by animal inoculation.	Fluid is sterile.

Specific Gravity.—The specific gravity is usually determined only approximately with the urinometer. In filling the cylinder, special care is required to avoid bubbles on the surface because of the albuminous nature of the fluid. The determination should be made at once, before clotting of the fluid will have occurred. Because of the high temperature of the fluid immediately after withdrawal (approximately that of the body), a correction of the urinometer reading should be made. Most instruments are standardized for a temperature of 15° C. For each three (3) degrees C. above this temperature the specific gravity is depressed 0.001. The result is that the reading obtained on a perfectly fresh fluid is too low. (It must be remembered, however, that the values obtained with the clinical urinometer are, at best, only approximately correct.)

Albumin Content.—Albumin content refers to the entire coagulable protein. For clinical use, the best method of determining the quantity of the coagulable protein is Tsuchiya's modification of the Esbach method (p. 31). It is necessary to dilute the fluid to such an extent that the reading in the Esbach tube will be 4 or less; in other words, the dilution must reduce the albumin below 0.4 per cent. With higher percentages of protein the method is subject to considerable error. The diluted fluid is weakly acidified with acetic acid, and the determination is carried out in the same manner as with urine. The reading at the end of 24 hours is multiplied by the dilution, and the result is the number of grams of coagulable protein per 1,000 c.c. of fluid.

Tsuchiya's method is less accurate with puncture fluids than with urine.[1]

Accurate determinations of the coagulable protein may be made in connection with the estimation of the nonprotein nitrogen (see above). Five c.c. of the puncture fluid (before clotting has occurred) are measured by means of a pipette into each of two Kjeldahl flasks. To the fluid in each flask add about 15 c.c. of concentrated sulphuric acid, about 0.2 gm. of copper sulphate crystals, and about 10 gm. of potassium sulphate. The usual Kjeldahl determination of total nitrogen is now made (p. 18). The distillate is collected in 50 c.c. of $\frac{N}{10}$ sulphuric acid. The total nitrogen in grams per cent is calculated. From this the incoagulable nitrogen in grams per cent is subtracted. The difference between the two, multiplied by the factor 6.25, gives the coagulable protein in grams per cent.

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[1] Mattice, A. F. Personal communication.

A Protein Precipitable in the Cold by Dilute Acetic Acid.—Runeberg [2] was the first to apply to the diagnosis of puncture fluids Paijkull's observation on the presence of a protein precipitable by dilute acetic acid in the cold. He found that *inflammatory exudates* and those resulting from *malignant neoplasms* of the serous membranes usually contain this body in abundance, whereas *transudates* do not, or at most possess it only in traces. His observations have been confirmed by Umber,[3] Stähelin,[4] and others. Umber has designated the body in question "serosamucin."

Method.—About 10 c.c. of the fluid in a test tube are treated with a few drops of dilute acetic acid (3 per cent) until the reaction becomes acid to litmus. A positive reaction is denoted by the appearance of a rather marked cloudiness. A slight cloud is of no significance, and is seen in transudates frequently. Previous clotting of the fluid apparently does not interfere with the test. A great excess of acetic acid may redissolve the precipitate, and is, therefore, to be avoided.

Cytology.—As is the case with the blood leukocytes, cells are occasionally seen in exudates and transudates which cannot be definitely classified, but, for the most part, the cells of puncture fluids may be placed in the following groups:

1. *Lymphocytes.*—Often cells similar to, and, doubtless, identical with, the small lymphocytes of the blood are numerous in puncture fluids. They are characterized by their small size, relatively large nucleus, surrounded by a narrow rim of protoplasm. Frequently the protoplasm is so scanty that the nucleus appears to be, and sometimes is, naked. All transitions in size from the lymphocyte to the endothelial cell may be observed.

2. *Endothelial Cells.*—These are usually very large cells, with abundant protoplasm and one or more round or oval nuclei, rather poor in chromatin. Frequently several cells are seen *en masse.* The size of the cells, as well as their shape, is variable; smaller forms are not infrequent. The cytoplasm may exhibit degenerative changes.

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[2] Runeberg, J. W. "Von der diagnostischen Bedeutung des Eiweissgehaltes in pathologischen Trans- und Exsudaten." *Berlin. klin. Wchnschr.*, 1897, XXXIV, 710.

[3] Umber, F. (*a*) "Ueber autolytische Vorgänge in Exsudaten." *München. med. Wchnschr.*, 1902, XLIX, 1169. (*b*) "Zum Studium der Eiweisskörper in Exsudaten." *Ztschr. f. klin. Med.*, 1903, XLVIII, 364.

[4] Stähelin, R. "Ueber den durch Essigsäure fällbaren Eiweisskörper der Exsudate und des Urins." *München. med. Wchnschr.*, 1902, XLIX, 1413.

3. *Polynuclear neutrophile cells* are similar to those of the blood, which is their source. They are often well preserved, but degenerations are common both in protoplasm and nucleus. The polymorphous nucleus may come to resemble a single round nucleus.

4. *Eosinophile cells* are much less commonly met with than any of the foregoing. They may, at times, form a striking feature of the cell picture, constituting 50 per cent or more of the cells. The conditions in which they have been found are varied, and, as yet, no diagnostic value can be assigned to them in pleural exudates. They have been observed by Rist and de Peffel,[5] with mast cells, in the exudates following *artificial pneumothorax.* The same authors also find that these cells occasionally predominate in *exudates of recent formation,* later giving way to lymphocytes. In some cases of *spontaneous pneumothorax,* they report similar findings. In several cases of *pleural effusion complicating lobar pneumonia,* eosinophiles have been conspicuous in the fluid; one case has been reported by Bayne-Jones,[6] who reviewed the literature. He states that pleural eosinophilia has been found following *trauma, sepsis, typhoid fever, syphilis, polyarthritis, nephritis, pulmonary gangrene, hemorrhagic infarct* of the lung, *endothelioma, septic endocarditis, gonococcal sepsis, myocarditis, cardiac insufficiency, puerperal sepsis, neoplasm,* and *influenza.*

5. *Mast cells* (*polynuclear basophiles*) are uncommon in puncture fluids. When found, they are usually associated with an increase in the percentage of the eosinophiles, as noted by Rist and de Peffel.[7]

6. *Tumor cells,* as such, are not recognizable. The presence of many mitotic nuclei, however, as Dock[8] was among the first to point out, is highly suggestive. Fragments of tissue, when obtained, should be hardened and studied in section.

7. *Red blood corpuscles,* often well preserved, though frequently crenated or otherwise degenerated, are often found in puncture fluids, particularly when of *tuberculous* or *malignant* origin.

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[5] Rist,E., and de Peffel. *Bull. Soc. d'Études scient. sur la Tuberculose.* 1914, p. 53. (*Abst. in Arch. des Mal. du Coeur,* etc., 1915, VIII, 229.)

[6] Bayne-Jones, S. "Pleural eosinophilia." *Johns Hopkins Hosp. Bull.,* 1916, XXVII, 12.

[7] Rist, E., and de Peffel, *op. cit.*

[8] Dock, G. "Cancer of the stomach in early life and the value of cells in effusions in the diagnosis of cancer of the serous membrane." *Amer. Jour. Med. Sci.,* 1897, CXIII, 655. *Also* Warren, L. F. "The diagnostic value of mitotic figures in the cells of serous exudates." *Arch. Int. Med.,* 1911, VIII, 648.

Method of Obtaining Cells.—The fluid should be centrifugalized at high speed before clotting has occurred. The cells are then removed from the bottom of the centrifuge tube with a pipette, spread on glass slides, dried, fixed, and stained. One of the Romanowsky stains, Jenner's stain, or hematoxylin and eosin may be employed.

In case the fluid cannot be centrifugalized immediately after its withdrawal from the body, about 10 c.c. of it should be discharged into an equal volume of 1 per cent sodium fluorid solution to prevent clotting. For the sodium fluorid there may be substituted a 1.5 per cent solution of sodium citrate in 0.85 per cent sodium chlorid; or a few small crystals of potassium oxalate may be added. Examination of the cells should be made within a day.

ANIMAL INOCULATION

Tubercle bacilli may be sought in exudates, but generally without success when one employs the direct methods of examination. By means of animal inoculation, it has been demonstrated that the great majority of serous exudates of the pleural cavity are tuberculous. About *15 c.c.* of the fluid are injected into the peritoneal cavity of a guinea pig, using aseptic precautions. In case the injection cannot be made at once, a small amount of sodium citrate should be added to the fluid to prevent clotting. Better results are obtained when large quantities of fluid are injected. It has been recommended that 10 to 50 c.c. be used weekly, until about 300 c.c. of exudate have been injected. Young animals are somewhat more susceptible to infection. After an interval of about six weeks the animal is sacrificed, and autopsy is performed, to determine the presence or absence of tuberculous lesions. Should the animal die before the six weeks have passed, autopsy is done at once. Using the large quantities of fluid, le Damany has demonstrated the tuberculous character of all but four in fifty-five cases of primary pleurisy, or 93 per cent; using 15 c.c., Eichhorst found that more than 62 per cent of cases were tuberculous, while less than 10 per cent of the animals developed tuberculous lesions when only 1 c.c. of the exudate was injected (Osler).

CEREBROSPINAL FLUID [9]

Lumbar puncture often yields only a few cubic centimeters of cerebrospinal fluid, though at times 50 c.c. or more may be safely withdrawn. The determination of specific gravity, which is normally between about 1.006 and 1.010, is of little importance clinically. The character of the fluid, the number and kind of cells, the globulin content, the bacteriological and immunological findings are the chief points of interest.

Physical Characteristics.—1. *Clear Fluids.*—*Normally,* the cerebrospinal fluid is perfectly clear and transparent, like water; it may have the same appearance in certain diseases. Thus, in *cerebrospinal lues,* in many *chronic meningitides,* in *meningismus,* in *uremia,* in *diabetic coma,* in *infantile paralysis,* in *lethargic encephalitis,* and in the *earliest stages of acute meningitides* clear fluids are encountered. In connection with most *chronic noninfectious organic lesions* of the central nervous system, the fluid is normal in appearance and also in other respects.

2. *Cloudy Fluids.*—Often the fluid is cloudy or turbid. When slight, the cloudiness is best demonstrated by agitating the tube containing the fluid. A similar appearance may be due to small quantities of blood which are present as a result of the puncture wound. Differentiation is made by examination of the sediment obtained by centrifugalization of the fluid. If the cloudiness is due to an *acute meningeal inflammation,* leukocytes will predominate, whereas red cells will be more numerous if the cloud is due to admixture of blood. Furthermore, in the latter instance, the usual tendency is for the fluid to become less cloudy in successive portions; collection of the fluid in three test tubes is often helpful.

3. *Purulent Fluids.*—Such fluids require no special description. The color is usually grayish, at times yellowish, and the degree of the turbidity is dependent upon the number of cells in the fluid. Purulent fluids are found in association with *acute meningitides.*

4. *Bloody Fluids.*—The appearance of the fluid is dependent upon the amount of blood present. It is important to differentiate between a true hemorrhagic fluid and one in which there is an admixture of

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[9] "The Physiology and Pathology of the Cerebrospinal Fluid," by Wm. Boyd, N. Y., 1920, is an excellent reference work, to which the special investigator is referred.

blood from the puncture wound. Laroche[10] mentions the following differential points:

a. In the *three tube test,* one collects the fluid in three successive tubes. If the bloody appearance is the same in all three, it indicates a hemorrhagic lesion. On the other hand, when the appearance of the fluid differs in the three tubes, the likelihood is that a vein has been punctured.

b. Centrifugalization of the fluid yields a bloody sediment. In the case of a *hemorrhagic lesion* the supernatant fluid has a *yellowish color,* due to destruction of red cells, which begins within a few hours after the blood is extravasated. On the contrary, *admixture of blood* at the time of the puncture gives one a *clear, colorless fluid* after centrifugalization. However, this difference may fail during the first twenty-four hours of an acute hemorrhagic lesion.

c. A hemorrhagic cerebrospinal fluid *does not coagulate.* A cerebrospinal fluid with admixture of blood at the time of the puncture coagulates, even after centrifugalization.

Bloody fluids may be found after *fracture* of the skull or of the vertebrae, after *cerebral hemorrhage,* in certain cases of *pachymeningitis,* in connection with *acute lesions* involving the meninges.

5. *Yellow Fluids.*—A yellow color (*xanthochromia*) in the fluid is observed either in the course of *meningeal hemorrhages,* of *compressions of the cord,* or with certain *cerebral tumors.* It has also been observed in the course of certain cases of meningitis treated by *serum injections* and in association with *jaundice.* The yellow color is, therefore, the result of diverse causes. In icterus, the color is due to the bile pigments. After serum injections, the yellow color is the result of unabsorbed serum; this mixture of spinal fluid and serum coagulates spontaneously.

In the other instances (hemorrhages, compressions, tumors), the yellow color results either from resorption of small hemorrhages or from the escape of blood serum into the subarachnoid space from circulatory disturbances. In the first instance, the fluid does not coagulate, whereas in the second it coagulates spontaneously and very rapidly—*massive coagulation with xanthochromia.* In some cases, the two processes, that is, capillary hemorrhages and transudation of serum, may be associated, as, for example, in the case of tumors of the cerebrum or cord.

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[10] *Examens de Laboratoire du Médecin Practicien.* Paris, 1919, 121.

Cells of the Cerebrospinal Fluid.—The cerebrospinal fluid normally contains very few cells. Indeed, some observers have reported no cells in the normal fluid, though such a finding is probably exceptional. From 1 to 7 cells per cubic millimeter has been given as the normal limit.[11] More than 10 cells per c. mm. is pathological.[12] The cells are chiefly lymphocytes. In disease pus cells may be numerous; endothelial cells, eosinophiles, mast cells, and erythrocytes may be seen.

Method of Counting the Cells.—A saturated aqueous solution of methyl violet (5*B*) is drawn up in the tube of a 1:100 blood pipette, until it has filled four of the decimal divisions on the capillary tube, and then the pipette is filled with the fresh spinal fluid, which should have been well shaken just before making the dilution to insure a uniform suspension of the cells. The leukocytes are stained violet. The pipette is shaken three minutes, and the count is then made with the blood-counting chamber, observing the usual technic for counting blood. Emerson[13] recommends the use of the leukocyte pipette. The capillary tube is filled to the mark 0.5 with Unna's polychrome methylene blue, then to the mark 11 (or 21) with the fresh cerebrospinal fluid.

At times, in performing lumbar puncture, blood becomes mixed with the cerebrospinal fluid, the result being that the blood leukocytes raise the count of the cells of the cerebrospinal fluid. In such case the red cells in the fluid are counted, then both red and white cell counts of the blood are made. From the latter one obtains the relative number of red and white cells which have been introduced into the cerebrospinal fluid, and the count of the latter may be corrected. In making such correction it is essential that there shall have been no laking of the erythrocytes in the cerebrospinal fluid.

Differential Count of the Cells.—The fresh fluid is centrifugalized, and the sediment is transferred with a pipette to clean glass slides, on which it is spread. The dried films are fixed and stained, usually with a Romanowsky stain. A differential count of the cells is then made[14] (see p. 304).

[11] Rous, F. P. "Clinical studies of the cerebrospinal fluid, with especial reference to pressure, protein-content, and the number and character of the cells." *Amer. Jour. Med. Sci.*, 1907, CXXXIII, 567.

[12] McCampbell, E. F., and Rowland, G. A. "Studies on the clinical diagnosis of general paralysis of the insane." *Jour. Med. Research*, 1910, XXII, 169.

[13] Emerson, C. P. *Clinical Diagnosis*, 1911, 700.

[14] Szecsi, S. "Neue Beiträge zur Cytologie des Liquor cerebrospinalis: Ueber Art und Herkunft der Zellen." *Ztschr. f. d. ges. Neurol. u. Psychiat.*, 1911, VI, 537.

Bacteriology of the Cerebrospinal Fluid

Micrococcus Intracellularis Meningitidis (Meningococcus).—In cases of epidemic cerebrospinal meningitis, this organism is usually demonstrable in the spinal fluid. The fluid is generally cloudy or frankly purulent, though in the earliest stages of the disease or as a result of serum treatment, a clear fluid may be obtained. The stained smear of

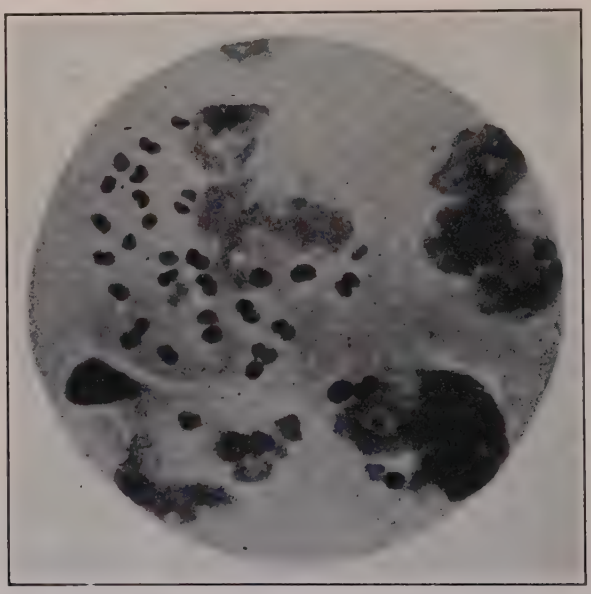

Fig. 99.—Micrococcus intracellularis meningitidis in the Spinal Fluid ×3000 (after Wm. B. Wherry; photomicrograph by Chas. Goosmann).

the sediment obtained by centrifugalization of the fluid, even in the latter instances, usually shows a preponderance of pus cells.

Morphology.—In the stained specimen of spinal fluid, the meningococci are found both intra- and extracellularly and resemble the gonococci (Fig. 99). They are usually in diplococcus groups, sometimes as tetrads or even in larger agglomerations.[15] The diplococci are flattened on the sides facing each other, having the biscuit or coffee-bean form of the gonococcus. The variation in the size of the cocci in the same smear is a noticeable feature and of some diagnostic importance. The fact

[15] From Zinsser, H. *A Textbook of Bacteriology* (5th ed.), 1922, 515.

must be remembered that meningococci in the spinal fluid may undergo solution, with the result that spinal fluid full of pus cells may contain very few recognizable organisms.

Staining.—The organisms are Gram-negative. With Neisser's stain, metachromatic granules in the center of the cell bodies may be demonstrated, a point of some value in differentiating meningococcus from gonococcus. The sediment of the spinal fluid may be stained with Jenner's stain or with one of the Romanowsky stains (Wright's, Leishman's, Wilson's, etc.).

Cultivation.—Tubes of blood-agar or ascites fluid agar are inoculated with the spinal fluid. The organisms grow best when about 10 per cent of the air has been replaced by carbon dioxid.

Bacillus tuberculosis is demonstrable in the great majority of instances of tuberculous meningitis. Several cubic centimeters of the fluid are placed in a sterile test tube, which is put in the refrigerator for 12 to 24 hours. A very delicate, filmy clot forms. This enmeshes the majority of the tubercle bacilli. The clot is then transferred to a clean slide, spread out into a thin layer with needles, air-dried, and fixed with heat. The specimen is then stained by the Ziehl-Neelsen method for tubercle bacilli. With this method Hemenway [16] has demonstrated the organisms in a large series of cases.

A second method of searching for the tubercle bacillus, which gives less constant results, is to centrifugalize the fluid at high speed until the sediment is thrown down (about 2,000 revolutions for ½ hour); it is then transferred to slides and examined in the usual manner for tubercle bacilli.

In case of negative findings, several cubic centimeters of the fluid may be injected into the peritoneal cavity of a guinea pig (see p. 410).

Other Organisms in the Spinal Fluid.—Purulent meningitis, primary in the sense that the portal of entry is obscure, is quite often due to *pneumococci* or *B. influenzae* and related organisms. The *staphylococcus* and the *Streptococcus pyogenes* are more often found secondary to trauma about the head or to phlebitis. Secondary localization in the meninges or brain may follow infection of other parts of the body with *B. typhosus, B. paratyphosus, B. coli, B. pestis, B. mallei, B. diphtheriae, B. anthracis,* the *gonococcus,* and *Actinomyces bovis.* Cases of meningeal

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[16] Hemenway, J. "The constant presence of tubercle bacilli in the cerebrospinal fluid of tuberculous meningitis." *Amer. Jour. Dis. Child.,* 1911, I, 37.

CEREBROSPINAL FLUID IN DISEASE

Disease	Increased Pressure	Average cells in fluid per cu. mm.	Predominating cells in fluid	Globulin	Bacteria
Acute cerebrospinal meningitis	+	50 (early) to several thousand	Polynuclear neutrophiles	+	Meningococcus. Pneumococcus. Streptococcus.
Tuberculous meningitis	+	50 to 250 or more	Lymphocytes, 90 per cent (Tashiro's test)	+	Tubercle bacilli.
Acute syphilitic meningitis	+	100 to 500, rarely more	Lymphocytes, 60–80 per cent (Emerson)	+	None
Acute anterior poliomyelitis	±	15–1200	Lymphocytes and * polynuclear neutrophiles	+	None ?
Epidemic (lethargic) encephalitis	+	Normal to 10–100; rarely higher	Lymphocytes and polynuclear neutrophiles	−	None
Mumps, with meningeal complications *	±	100 to several thousand	Lymphocytes	−	None
Herpes zoster *	−	50	Lymphocytes	−	None
Cerebral hemorrhage	+	100 to several thousand, or normal fluid	Erythrocytes or normal	+ from blood	None
Tabes dorsalis	−				
General paresis	±	25–70–100; rarely more	Lymphocytes	+	None
Cerebrospinal lues	±				
Meningismus (Serous meningitis)	+	Fluid normal		−	None
Uremia	+	Fluid normal. *Urea* 0.1–0.6. (Normal 0.01–0.06)	Lymphocytosis (Inconstant) †	−	None
Diabetic coma *	?	Fluid normal. *Sugar* increased up to 0.5 per cent. *Acetone* present in fluid, with coma or impending coma		−	None
Trichinosis *	?	Normal	*Embryos* in fluid	−	None

* Boyd, Wm., *loc. cit.* (p. 318).

† The earlier the stage of the disease, the higher the proportion of polynuclear cells, up to 80 or 90 per cent and the higher the cell count. Globulin increase is inversely proportional to cell count.

infection with *B. tuberculosis* usually run a more chronic course, though at times the clinical picture approaches that of acute meningitis.

Parasites in the Cerebrospinal Fluid.—In sleeping sickness, *trypanosomes* (see p. 348) may be found in the spinal fluid. About 10 c.c. of spinal fluid, placed in a sterile centrifuge tube, are centrifugalized at high speed for ten to fifteen minutes. Fresh preparations of the sediment are examined for the trypanosomes; the sediment is also stained for trypanosomes with Leishman's or Wright's stain. According to Castellani, the result of the examination is almost constantly positive in the sleeping sickness stage, but negative, as a rule, in the first stage of the disease. A history of residence in tropical Africa, in addition to the clinical symptoms, will suggest the possibility of the infection.

Trichinella spiralis embryos have been reported in the spinal fluid in cases of trichinosis.

Globulin Content.—Globulin is the most important protein of the cerebrospinal fluid. It is often present in increased quantity in disease, and may be detected by the following tests. Fluids with an admixture of blood cannot be used, since the serum globulin vitiates the tests.

1. *Method of Noguchi.*[17]—To 0.1 c.c. of spinal fluid add 0.5 c.c. of a 10 per cent solution of *pure* butyric acid in 0.9 per cent sodium chlorid and boil briefly over the flame. Then add quickly 0.1 c.c. of $\frac{N}{1}$ sodium hydroxid and again boil for a few seconds. With an increase of globulin a coarse granular or flocculent precipitate appears, usually within 10 to 20 minutes. If the precipitate does not appear within this time, the test tube is set aside and observed at the end of three hours. Normal fluids or those in which globulin is not increased give rise to a slight and uniform opalescence only, and no coarse precipitate forms, even after several hours. In case of an ambiguous reaction, Noguchi advises a repetition of the test with 0.2 c.c. of spinal fluid.

2. *Method of Ross and Jones.*[18]—Two c.c. of a saturated aqueous solution of ammonium sulphate are placed in a test tube, and 1 c.c. of the spinal fluid is gently run onto the surface of it, while the tube is

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[17] Noguchi, H. "The relation of protein, lipoids, and salts to the Wassermann reaction." *Jour. Exp. Med.*, 1909, XI, 84. Noguchi, H., and Moore, J. W. "The butyric acid test for syphilis in the diagnosis of metasyphilitic and other nervous disorders." *Ibid.*, 1909, XI, 604. *See also* Strouse, S. "The diagnostic value of the butyric acid test (Noguchi) in the cerebrospinal fluid." *Jour. A. M. A.*, 1911, LVI, 1171.

[18] Ross, G. W., and Jones, E. "On the use of certain new chemical tests in the diagnosis of general paralysis and tabes." *British Med. Jour.*, 1909, I, 1111.

inclined, so as to form a layer above the ammonium sulphate. The formation of a thin, grayish white ring at the line of contact of the two fluids constitutes a positive reaction. The precipitate should form within three minutes. Within one-half hour it may be observed that the surface of the ring shows a delicate mesh appearance resembling a fine cobweb. The ring should be looked for with indirect illumination, the tube being held against a dark background with the eye at a right angle to the source of light. It is essential that the ammonium sulphate be quite neutral, not acid, and that the solution be saturated.

3. *Pándy's Test.*[19]—To 1 c.c. of concentrated aqueous solution of carbolic acid (about 7 per cent, that is, 1 part of carbolic acid crystals to 15 parts of distilled water), add a drop of cerebrospinal fluid. An excess of globulin is indicated by a bluish white cloud.

4. *Tashiro's Test.*[20]—Into each of two small test tubes of equal diameter place like quantities (1 c.c.) of cerebrospinal fluid. One c.c. of 3 per cent sulphosalicylic acid is added to the one, and 1 c.c. of 1 per cent mercuric chlorid is added to the other. The tubes are then set aside for twenty-four hours to allow the precipitate to settle. Normal fluids give only a very slight precipitate. In *acute meningitides* sulphosalicylic acid gives much the heavier precipitate (about 2 to 3 times that obtained with bichlorid), whereas in *tuberculous meningitis* the findings are reversed, the precipitate with bichlorid being much more abundant than that with sulphosalicylic acid.

No turbidity is produced with mercuric chlorid with *normal* fluids. With *pathological* fluids, there is no precipitate at first, but in 12 to 24 hours a gelatinous precipitate settles out. With normal fluid, the sulphosalicylic acid sediment usually measures 1 to 3 mm. in height, the mercuric chlorid 2 to 4 mm. in height.

The Wassermann Reaction in Cerebrospinal Fluid.—In syphilitic and metasyphilitic disease of the central nervous system the fluid obtained at lumbar puncture may yield a positive Wassermann reaction (see p. 359).

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[19] Pándy, K. "Ueber eine neue Eiweissprobe für die Cerebrospinalflüssigkeit." *Neurol. Centralblatt,* 1910, XXIX, 915.

[20] Tashiro, S. and Levinson, A. "Alkaloidal and metallic precipitation of cerebrospinal fluid in the diagnosis of meningitis." *Jour. Infect. Dis.,* 1917, XXI, 554.

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